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# Control Of Systemic Lipid Metabolism By Adipocyte Mtor Signaling

## Abstract

Pharmacological agents targeting the mTOR complexes are used clinically as immunosuppressants and anticancer agents, and can extend lifespan in model organisms. An undesirable side effect of these drugs is hyperlipidemia. Raptor and Rictor are essential component of mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) and 2 (mTORC2), respectively. Despite multiple roles that have been described for mTOR complex 1 (mTORC1) in lipid metabolism, the etiology of hyperlipidemia remains incompletely understood. The objective of this study was to determine the influence of adipocyte mTORC1 signaling in systemic lipid homeostasis in vivo. We characterized systemic lipid metabolism in mice lacking the mTORC1 subunit raptor (RaptoraKO), the key lipolytic enzyme ATGL (ATGLaKO), or both (ATGL-RaptoraKO) in adipocytes. Mice lacking mTORC1 activity in adipocytes failed to completely suppress lipolysis in the fed state and displayed prominent hypertriglyceridemia and hypercholesterolemia. Blocking lipolysis in adipose tissue restored normal levels of triglycerides and cholesterol in the fed state, as well as the ability to clear triglycerides in an oral fat tolerance test. Unsuppressed adipose lipolysis in the fed state interferes with triglyceride clearance and contributes to hyperlipidemia. Adipose tissue mTORC1 activity is necessary for appropriate suppression of lipolysis and for the maintenance of systemic lipid homeostasis. Loss of mTORC1 signaling in adipose is sufficient to disrupt lipid homeostasis, resulting in hyperlipidemia caused by unrestrained lipolysis. However, studies to date examining the role of deletion of this complex, complex 2 or both in adipose tissue in combination with rapamycin have yet to be investigated. Here, we report the consequences of Raptor, Rictor or both deleted specifically in mature adipocytes driven by Adiponectin-Cre (RaptoraKO, RictoraKO, Raptor-RictoraKO). Concordant with the RaptoraKO mice, RictoraKO mice display pronounced hyperlipidemia and both KO models have a further increase in plasma lipids with rapamycin treatment. Genetic inhibition of lipolysis in mice with loss of mTORC1 (ATGL-RaptoraKO) treated with rapamycin prevents the further increase in plasma lipids seen in RaptoraKO mice treated with rapamycin. We propose a hypothetical mechanism that in the fed state, rapamycin inhibition of adipose mTORC1 leads to decreased C/EBP transcriptional activity. This decreased transcriptional activity results in decreased expression of perilipin and subsequent unrestrained lipolysis, leading to hyperlipidemia. Here we show that enhanced lipolysis upon refeeding increases plasma triglyceride levels in the context of rapamycin treatment and that both complexes are involved in regulating this lipolytic process. However, mTORC1, not mTORC2, is required for proper adipocyte lipolysis to maintain circulating plasma lipid levels. Additionally, we provide evidence that loss of adipocyte mTOR signaling is not solely responsible for the rapamycin induced hyperlipidemia.

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Lauren M. Paoella

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*For my parents, Dr. Arthur C. Paolella and Judy L. Paolella*

# ABSTRACT

## CONTROL OF SYSTEMIC LIPID METABOLISM BY ADIPOCYTE MTOR SIGNALING

Lauren M. Paoletta

Joseph A. Baur, Ph.D.

Pharmacological agents targeting the mTOR complexes are used clinically as immunosuppressants and anticancer agents, and can extend lifespan in model organisms. An undesirable side effect of these drugs is hyperlipidemia. Raptor and Rictor are essential components of mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) and 2 (mTORC2), respectively. Despite multiple roles that have been described for mTOR complex 1 (mTORC1) in lipid metabolism, the etiology of hyperlipidemia remains incompletely understood. The objective of this study was to determine the influence of adipocyte mTORC1 signaling in systemic lipid homeostasis *in vivo*. We characterized systemic lipid metabolism in mice lacking the mTORC1 subunit raptor (Raptor<sup>akO</sup>), the key lipolytic enzyme ATGL (ATGL<sup>akO</sup>), or both (ATGL-Raptor<sup>akO</sup>) in adipocytes. Mice lacking mTORC1 activity in adipocytes failed to completely suppress lipolysis in the fed state and displayed prominent hypertriglyceridemia and hypercholesterolemia. Blocking lipolysis in adipose tissue restored normal levels of triglycerides and cholesterol in the fed state, as well as the ability to clear triglycerides in an oral fat tolerance test. Unsuppressed adipose lipolysis in the fed state interferes with triglyceride clearance and contributes to hyperlipidemia. Adipose tissue mTORC1 activity is necessary for appropriate suppression of lipolysis and for the maintenance of systemic lipid homeostasis. Loss of mTORC1 signaling in adipose is sufficient to disrupt lipid homeostasis, resulting in hyperlipidemia caused by unrestrained lipolysis. However, studies to date examining the role of deletion of this complex, complex 2 or both in adipose tissue in combination with rapamycin have yet to be investigated. Here, we report the consequences of Raptor, Rictor or both deleted specifically in mature adipocytes driven by Adiponectin-Cre (Raptor<sup>akO</sup>, Rictor<sup>akO</sup>, Raptor-Rictor<sup>akO</sup>). Concordant with the Raptor<sup>akO</sup> mice, Rictor<sup>akO</sup> mice display pronounced hyperlipidemia and both KO models have a further increase in plasma lipids with rapamycin treatment. Genetic inhibition of lipolysis in mice with loss of mTORC1 (ATGL-Raptor<sup>akO</sup>) treated with rapamycin prevents the further increase in plasma lipids seen in Raptor<sup>akO</sup> mice treated with rapamycin. We propose a hypothetical mechanism that in the fed state, rapamycin inhibition of adipose mTORC1 leads to decreased C/EBP $\alpha$  transcriptional activity. This decreased transcriptional activity results in decreased expression of perilipin and subsequent unrestrained lipolysis, leading to hyperlipidemia. Here we show that enhanced lipolysis upon refeeding increases plasma triglyceride levels in the context of rapamycin treatment and that both complexes are involved in regulating this lipolytic process. However, mTORC1, not mTORC2, is required for proper adipocyte lipolysis to maintain circulating plasma lipid levels. Additionally, we provide evidence that loss of adipocyte mTOR signaling is not solely responsible for the rapamycin induced hyperlipidemia.

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## **CHAPTER 1: General Introduction**

## 1.1 Rapamycin

### 1.1.1 Discovery of Rapamycin

Rapamycin, an antifungal antibiotic, was discovered in 1975 (Vezina et al., 1975).

Cultures of a bacteria strain, *Streptomyces hygroscopicus*, were isolated from an Easter Island (Rapa Nui) soil sample. The active antifungal principle was isolated in a crystalline form and named rapamycin [Etymol.: Rapa- (Rapa Nui=Easter Island), -mycin].

Rapamycin is a white, crystalline solid with a melting point range of 183-185°C, and is poorly soluble in water but readily soluble in ethanol, methanol, dimethylsulfoxide and other organic solvents. Although the first chemical structure was proposed in 1980 (Findlay et al., 1980), the structure was not confirmed until total organic synthesis almost ten years later (Hayward et al., 1993; Nicolaou et al., 1993; Romo et al., 1993).

Structural characterization showed rapamycin to be a mixture of two conformational isomers because of the cis-trans rotation in the macrolidic lactone ring. This molecule was discovered to have two structural domains, an effector domain and a binding domain that facilitates the binding of rapamycin to FKBP12 (Figure 1.1) (Bierer et al., 1990; Dumont et al., 1990a; Dumont et al., 1990b).

Rapamycin, a strong inhibitor of yeast growth, is mainly active against all strain of the yeast, *Candida albicans*. Early studies demonstrated that rapamycin at a concentration of 1.0 µg/ml did not inhibit the growth of this yeast during the first hour after addition to media, however, a lower concentration of 5.0 ng/ml completely prevented growth after addition to media. It prevents systemic and vaginal candidosis in mice with no acute toxic side effects (Baker et al., 1978). Several other yeasts and dermatophytes, filamentous fungi, are also sensitive to inhibition but to a lesser degree. No antibacterial

activity was demonstrated (Baker et al., 1978; Vezina et al., 1975). Initial characterization of rapamycin was shown to inhibit synthesis of nucleic acids, proteins and lipids, as well as promoting degradation of macro molecules (Singh et al., 1979).

Two years after the discovery of rapamycin, immunosuppressive and anti-tumor properties were discovered (Douros et al., 1981; Eng et al., 1984; Martel et al., 1977). Although initially discovered as an antifungal metabolite, studies later revealed inhibitory effects against production of humoral IgE. Studies also revealed preventive effects of rapamycin in animal models of experimental autoimmune encephalitis and adjuvant arthritis, two human autoimmune diseases (Martel et al., 1977). These studies demonstrated the first recognized effects of rapamycin's immunosuppressive activity. Later studies showed prolongation of survival and prevention of the progression of systemic lupus erythematosus in a MLR/lpr mouse model and exposed rapamycin's inhibitory effects against arthritis in rats (Kahan et al., 1991).

In 1990, studies were able to identify the molecular target of rapamycin. Rapamycin binds to intracellular receptors called FK506 binding proteins (FKBPs). More specifically, it forms a gain-of-function complex with the peptidyl-prolyl-isomerase (PPI) FKBP12. Due to the conformation of rapamycin, it is believed to be energetically favorable for FKBP12 binding (Van Duyne et al., 1991). FKBP12, an important FK506 binding protein, is a 12 kDa protein present in the cytosol that interacts with the immunosuppressant FK506, to inhibit cell growth and proliferation (Dumont et al., 1990a; Dumont et al., 1990b; Schreiber, S. L., 1991). Results from these studies showed that rapamycin and FK506 compete for interaction with FKBP12.



### *1.1.2 Identification of TOR*

Identification of the targets of the FKBP12-rapamycin complex helped gain insight into the molecular mechanisms of rapamycin's actions. Several genetic studies in yeasts identified two homologues of a protein named TOR, named target of Rapamycin (Heitman et al., 1991; Kunz et al., 1993). TOR has emerged as a critical regulator of the protein synthesis and cell growth in *C. elegans*, *Drosophila*, yeast, plants, and mammals. Unlike yeast, which contain two TOR genes, higher eukaryotes have only a single TOR gene (Crespo et al., 2005; Lee, S. et al., 2005).

Eukaryote TORs are large 280 kDa proteins and share 40%–60% sequence identity. They belong to a group of kinases known as the phosphatidylinositol kinase-related kinase (PIKK) family. PIKKs contain a carboxy-terminal serine/threonine protein kinase domain which is similar to the catalytic domain of phosphatidylinositol 3-kinases (PI3Ks). The FKBP12-rapamycin binding domain (FRB) is located at the amino-terminal region. Single amino acid substitutions in FRB of TOR1 and TOR2 prevents the binding and inhibition by the FKBP12-rapamycin complex. The amino-terminal portion of TOR contains tandem HEAT repeats for protein-protein interactions (Wullschleger et al., 2006).

Two separate genes TOR1 and TOR2 were mutated and loss of the functions of these two proteins mimicked the effects of rapamycin treatment (Heitman et al., 1991; Stan et al., 1994; Zheng et al., 1995). Purification of yeast TOR1 and TOR2 identified two functionally and structurally different complexes. TORC1, a rapamycin sensitive complex, and TORC2, a rapamycin insensitive complex (Loewith et al., 2002; Reinke et al., 2004). Yeast TOR complex 1 also contained Kog1, Tco89, and lethal with SEC13

protein 8 (Lst8) with TOR1 or TOR2 (Adami et al., 2007; Loewith et al., 2002; Reinke et al., 2004). Yeast TOR complex 2 also contained Avo1-3, Bit61, and Lst8 with TOR2 (Loewith et al., 2002; Wullschleger et al., 2005).

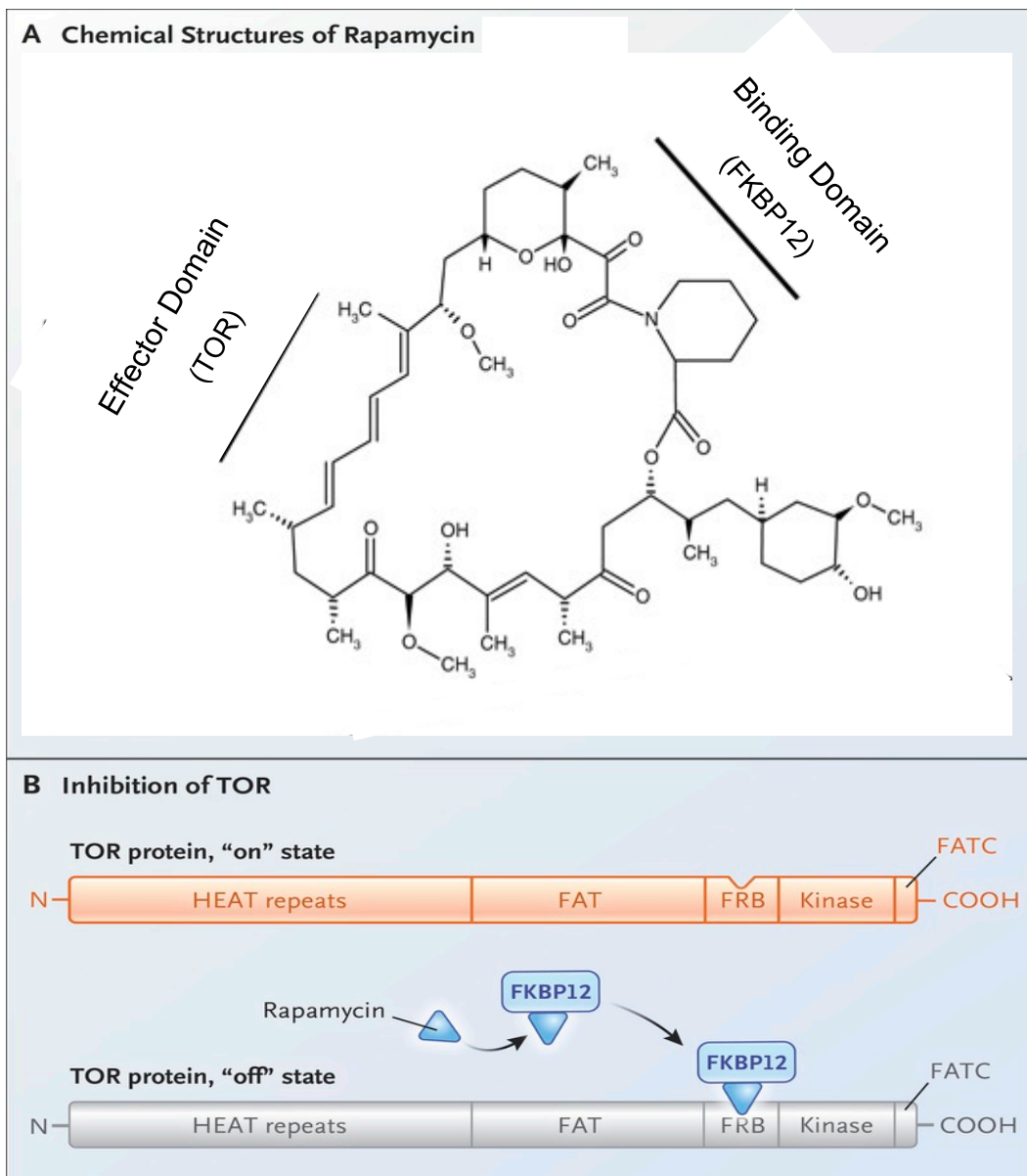
A few years after cloning of the yeast TOR gene, a mammalian cell ortholog was discovered after screening proteins that interacted with the FKBP12-rapamycin complex. Each group named the protein differently, rapamycin and FKBP12 target 1 (RAFT1), FKBP12-rapamycin associated protein (FRAP) or rapamycin target 1 (RAPT1) (Brown, E. J. et al., 1994; Chiu et al., 1994; Sabatini et al., 1994). It wasn't until 1995, when the gene was cloned that it was named mammalian target of Rapamycin (mTOR), based on the sequence homology with the yeast TOR proteins (Sabers et al., 1995). Although mammals have only one *MTOR* gene, it forms two distinct complexes, mTORC1 and mTORC2 (Saxton et al., 2017; Wullschleger et al., 2006).

The first component of mTORC1 identified was regulatory-associated protein of mTOR (Raptor) (Hara et al., 2002; Kim, D. H. et al., 2002). This protein is responsible for recruitment of substrates to mTORC1 by binding to the TOR signaling motif and for subcellular localization of mTORC1 (Nojima et al., 2003; Sancak et al., 2008; Schalm et al., 2003). Identification of Raptor-independent companion of mTOR (rictor) and mammalian stress-activated protein kinase-interacting protein (mSin1), lead to the existence of mTORC2 (Frias et al., 2006; Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004; Yang, Q. et al., 2006). Both complexes contain mammalian LST8 (mLST8), a positive regulator of the complexes function, DEP domain-containing mTOR-interacting protein (DEPTOR), an inhibitor of the complexes, and proline-rich

AKT substrate of 40 kDa (PRAS40), a mediator of growth factors and AKT signaling (Saxton & Sabatini, 2017; Wulfschleger et al., 2006; Wulfschleger et al., 2005).

**Figure 1-1. Rapamycin binding and inhibition of TOR.**

(A). Chemical structure and domains of rapamycin. Rapamycin (sirolimus) has a binding domain for FKBP12 and an effector Domain for TOR binding. common chemical moiety that binds to the FKBP12 protein. (B) Rapamycin first binds to FKBP12, then the protein–drug complex binds to the FKBP–rapamycin binding (FRB) domain on TOR to inhibit TOR proteins, Other conserved domains in TOR proteins include tandem HEAT repeats, a FAT domain, and a phosphoinositide 3-kinase–related kinase domain (kinase). Reproduced with permission from Manning BD, (2017), Copyright Massachusetts Medical Society (Manning, 2017).



### *1.1.3 Clinical Uses*

Rapamycin is clinically known as sirolimus. The earliest and most common clinical use of rapamycin is as an immunosuppressant to prevent kidney and cardiac rejection after transplantation. Rapamycin was first approved by the US Food and Drug Administration (FDA) in September 1999 and the European Commission in March 2000 for use in kidney transplantation. Rapamycin, and three analogs (CCI-779, RAD001 and AP23573) have been tested for treatment of human cancer.

Rapamycin represses activation of T cells upon association with an antigen presenting cell thereby strongly inhibiting proliferation of T cells. In this process, recognition of the antigen by the T-cell receptor activates the AKT pathway, leading to mTORC1 stimulation and subsequent initiation of cell growth and proliferation (Chi, 2012).

Rapamycin also alters dendritic cell maturation, migration, antigen presentation and apoptosis, thereby regulates antigen presenting cells (Thomson et al., 2009). This initially made Rapamycin a promising drug especially since it had a better adverse effect than commonly used immunosuppressing drugs. These drugs, such as Cyclosporine A and FK506, caused nephrotoxicity, which is not observed with rapamycin treatment. However, more recent data in patients treated primarily with rapamycin revealed proteinuria and an increased risk of early transplant rejection compared to calcineurin inhibitors, a steroid-sparing immunosuppressant used for over three decades in solid organ transplantation (Langone et al., 2012). Currently, in transplant patients that have developed secondary malignancies due to immune suppression, rapamycin is the first choice in treatment. In this context, rapamycin has displayed excellent effects as a result of its antiproliferative effects. In addition, rapamycin has been increasingly used in the

treatment of other disorders with immune system hyper activation. Clinical trials are have been carried out to explore the effect of rapamycin treatment in systemic lupus erythematosus, pemphigus, and uveitis.

Due to the poor water solubility of rapamycin, the development of two water-soluble rapamycin analogs (rapalogs), temsirolimus (2007) and everolimus (2009) gained FDA approval. In 2011, Everolimus was approved for use in progressive neuroendocrine tumors of pancreatic origin (PNET) and has been tested in clinical trials for use against advanced gastric cancer and advanced hepatocellular carcinoma. Clinical trials for temsirolimus have been done to assess the use in treating advanced neuroendocrine carcinoma (NEC), endometrial cancer and relapsed mantle cell lymphoma (MCL). Ridaforolimus, another rapalog, has been clinically tested for its use in patients with advanced bone and soft tissue carcinoma (Wander et al., 2011). However, since mTORC1 regulates certain negative feedback loops, rapalogs have has limited therapeutic for use in the treatment of major types of solid tumors.

#### *1.1.4 Anti-Aging*

The use of rapamycin as a lifespan extension agent has been extensively studied. The relationship between TOR and longevity was first shown in non-vertebrates. In yeast, deletion of SCH9, S6K homologue, or knockdown of TOR or *daf-15*, homologue of Raptor, then main mTORC1 protein member, in worms extended lifespan (Fabrizio et al., 2001; Jia et al., 2004; Vellai et al., 2003). In 2013, inhibition of mTOR was discovered to promote lifespan extension in nematodes and fruit flies (Lamming et al., 2013). Extension of lifespan has also been proven in mammals. 18-24 months of Rapamycin treatment in both male and female mice starting from either 9th or 20th

months of age extended both median and maximal lifespan (Harrison et al., 2009). A study in 2012 was designed to test age-dependent effects of mice treated with rapamycin from 9th to 21st month of age. These results demonstrated that age-related pathologies occur slower in mice treated with rapamycin (Wilkinson et al., 2012). In 2013, a study to test the age-independent effects of rapamycin was conducted. C57BL/6j mice were treated with rapamycin for 12 months starting at 4, 13 or 20 months of age. This study reported that extended lifespan in rapamycin treated mice but there was no effect on age related pathologies (Neff, F. et al., 2013). Based off these studies, it has since been concluded that rapamycin has an age-independent effect due to the improved effects in older and well as young mice.

#### *1.1.5 Metabolic Side Effects*

Recent advances in immunosuppressant drugs for solid organ transplant patients has decreased rates of rejection and increased life span of the transplant recipients. However, long term side effects including dyslipidemia and accelerated atherosclerosis still remain a problem. The most common clinical use of rapamycin is as an immunosuppressant to prevent kidney and cardiac rejection after transplantation. Sirolimus and other mTOR inhibitors such as everolimus and temsirolimus is commonly associated with adverse effects (Neff, G. W. et al., 2003). For cardiac transplants, hyperlipidemia occurs in 60% within one month and increases to 90% of patients within 10 years (Rudas et al., 1990; Taylor et al., 2008). For renal and liver transplants, hyperlipidemia occurs in over 50% of patients (Charco et al., 1999; Gisbert et al., 1997; Ramezani et al., 2007; Tse et al., 2004). In patients treated with rapamycin, serum triglycerides increase by 20% in over 50% of patients (Brattstrom et al., 1998; Firpi et al.,

2004; MacDonald et al., 2001; Trotter et al., 2001). In kidney transplant patients that develop rapamycin-induced hypertriglyceridemia, using an in vivo kinetic study, it has been shown that hypertriglyceridemia was mostly due to decreased VLDL catabolism (Hoogeveen et al., 2001). In that study, rapamycin treatment was associated with a 70% decrease in VLDL-ApoB100 catabolism and a decrease post-heparin LPL activity (Hoogeveen et al., 2001). Interestingly, it was shown that insulin upregulates LPL activity in a PI3K dependent manner and that this regulation was decreased by 60% in the presence of rapamycin (Kraemer et al., 1998). In addition, rapamycin treatment is associated with a 92% increase in ApoCIII, which is a potent inhibitor of LPL. In addition, rapamycin treatment induced a 42% increase in free fatty acids, suggesting augmented release of free fatty acids from the adipose tissue. This study suggest that this may be due to a reduction of the inhibition of the hormone-sensitive lipase by insulin(Morrisett et al., 2002).

The mechanisms by which sirolimus causes dyslipidemia remains unclear, but some studies have suggested that both an increase in VLDL synthesis (increased in apolipoprotein B) and a decrease in triglyceride hydrolysis (increased apolipoprotein CIII) (Hoogeveen et al., 2001; Morrisett et al., 2002; Tur et al., 2000). While other claims that the hyperlipidemia associated with rapamycin is not likely due to increased lipid hepatic synthesis but rather due to delayed peripheral clearance (Brown, N. F. et al., 2007). It has been postulated that this increase in free fatty acids may increase hepatic VLDL production, but such increase in VLDL production has not been clearly shown in human kinetic studies (Hoogeveen et al., 2001).



Mice studies have also observed adverse metabolic effects of rapamycin. KK/HIJ mice on a high fat diet showed aggravated glucose intolerance after treatment with rapamycin for six weeks (Chang et al., 2009b). Two weeks of rapamycin treatment in rats caused hyperlipidemia. This study suggested the hyperlipidemia was due to increased hepatic gluconeogenesis and impaired deposition of lipids in adipose tissue (Houde et al., 2010). A later study established that mTORC1 inhibition increased lifespan while mTORC2 inhibition resulted in insulin resistance (Lamming et al., 2012). Another study compared the effects of rapamycin on metabolism in mice after 2, 6 and 20 weeks of rapamycin treatment (Fang et al., 2013). The 2 week rapamycin treated group showed larger livers and smaller pancreas as compared to groups treated for longer. However, prolonged rapamycin treatment led to a decrease in adipose tissue. Rapamycin treatment affected the lipid profile in mice in proportional to the length of treatment. In addition, insulin was increased after 2 weeks of rapamycin treatment, causing the mice to become glucose intolerant and insulin resistant. Improved insulin sensitivity was reported in rapamycin treated mice treated for the longer durations of 6 weeks and 20 weeks (Fang et al., 2013).

## **1.2 Mammalian Target of Rapamycin (mTOR)**

### *1.2.1 Structure of mTOR*

Mammalian target of Rapamycin (mTOR) is a Ser/Thr protein kinase that belongs to the phosphatidylinositol 3-kinase-related kinases (PIKKs) superfamily which regulate cellular growth and differentiation (Ma, X. M. et al., 2009). mTOR is a 289 kDa protein comprised of 2549 amino acids. The structure of mTOR has six major domains. At the N-terminus are around 32 tandem HEAT (Huntingtin, elongation factor 3 (EF3), a subunit of PP2A,

and TOR) repeats, with each repeat comprised of ~ 40 amino acids. These repeats form a super helical structure and aids in protein-protein interaction (Andrade et al., 2001; Baretic et al., 2016). A ~600 residue FAT (named after FRAP, ATM, and TRRAP) domain, which consists of  $\alpha$ - $\alpha$  helical repeats that wraps partial around the kinase domain. A ~100 residue FRB (FKBP12 Rapamycin binding) domain that forms a four helix bundle. The FRB domain resides between the FAT and catalytic domains and acts as a gatekeeper for Rapamycin-FKBP12 binding (Choi et al., 1996; Yang, H. et al., 2013). A ~300 residue catalytic domain which forms a canonical two-lobed structure with key insertions in both the amino-terminal and the carboxy-terminal lobes. A ~40 residue LBE domain, which forms the binding site for mLST8. Lastly, a ~35 residue FAT-C domain at the carboxy terminus and is required for kinase activity (Takahashi et al., 2000). The six mammalian PIKKs contain three homologous domains; the FAT domain, catalytic domain, and the FAT-C domain (Andrade et al., 1995; Aylett et al., 2016; Bosotti et al., 2000; Yang, H. et al., 2013).

Although mTOR is expressed extensively in human tissues, levels are significantly higher in testis and skeletal muscle (Brown, E. J. et al., 1994; Chiu et al., 1994). Human mTOR shares 44% identity with the yeast TOR1 and 46% identity with the yeast TOR2. The 600 amino acids of their C-terminal region share the greatest sequence similarity of all three proteins, suggesting that mammalian mTOR and yeast TORs may have similar enzymatic activities. However, the other domains, which includes the N-terminal region, share little to no sequence similarity.

### 1.2.2 *mTOR Complexes: mTORC1 and mTORC2*

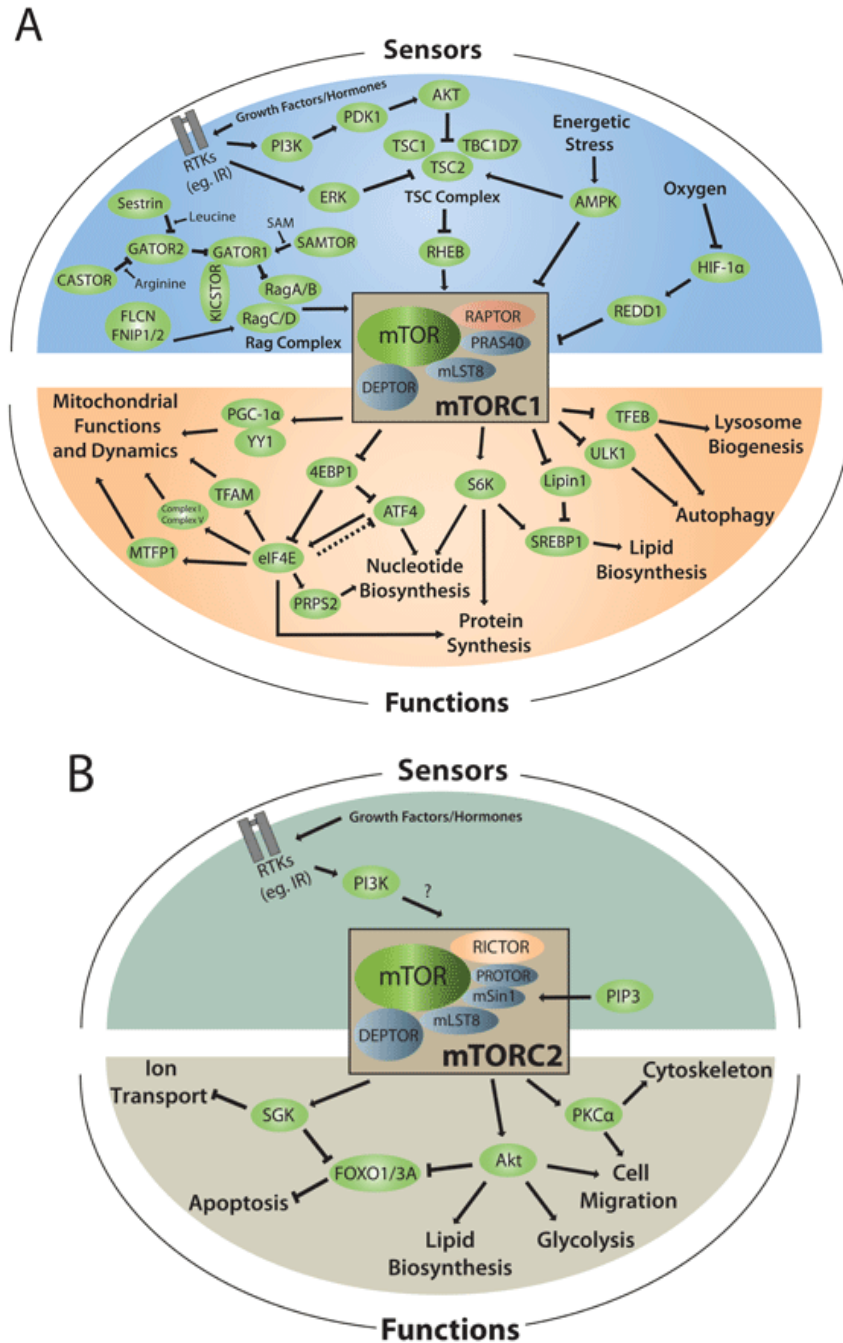
Unlike yeast, higher eukaryotes, have only one MTOR gene. However, it forms two distinct TOR complexes: mTORC1 and mTORC2. Efforts from many laboratories have revealed the molecular architecture comprising each complex (Wullschleger et al., 2006). mTOR integrates external nutrient cues to cellular growth and proliferation (Laplante et al., 2012). The two mTOR complexes have differential sensitivities to rapamycin – with mTORC1 more sensitive than mTORC2 (Sabatini, 2006). A notable initial contribution was the identification of regulatory-associated protein of mTOR (Raptor) as an mTORC1 component (Hara et al., 2002; Kim, D. H. et al., 2002). The existence of mTORC2 was demonstrated by the identification of Raptor-independent companion of mTOR (Rictor) and mammalian stress-activated protein kinase-interacting protein (mSin1) (Frias et al., 2006; Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004; Yang, Q. et al., 2006). Mammalian LST8 (mLST8) is a common core component of both mTORC1 and mTORC2 and positively contributes to their functions. DEP domain-containing mTOR-interacting protein (DEPTOR) also associates with mTORC1 and mTORC2, but acts to inhibit the two complexes. Another mTORC-associated protein is proline-rich AKT substrate of 40 kDa (PRAS40). PRAS40 may also mediate growth factor and AKT signals to mTORC1 and mTORC2.

As in yeast, the FKBP12–rapamycin complex directly binds to and inhibits mTORC1 kinase activity and function, but does not directly inhibit mTORC2. Studies of mTORC1 structure have provided insights into the inhibitory mechanism of action of rapamycin (Aylett et al., 2016; Stuttfeld et al., 2018; Yang, H. et al., 2013). Rapamycin, however, seems to inhibit only part of mTORC1 functions (Choo et al., 2008; Kang et al., 2013).

Rapamycin leads to a strong decrease in phosphorylation of ribosomal subunit S6kinase (S6K1) but only a slight decrease in phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP-1) (Kang et al., 2013; Yoon, S. O. et al., 2013). In most cell types, mTORC2 is insensitive to short term treatment with rapamycin (Oshiro et al., 2004; Sarbassov et al., 2004). However, long-term rapamycin treatment can abrogate mTORC2 signaling, probably due to the destabilization of the mTORC2 complex, in many cell lines and tissues *in vivo*, including hepatocytes, adipose tissue, skeletal muscle, heart, pancreases, liver, lung and spleen (Lamming et al., 2012; Sarbassov et al., 2006; Schreiber, K. H. et al., 2015).

## Figure 1-2. Upstream and downstream mTORC1 and mTORC 2.

(A). Upstream sensors and downstream function of mTOR complex 1. (B) Upstream sensors and downstream function of mTOR complex 2. Reproduced with permission from (Papadopoli et al., 2019).



### *1.2.3 Upstream mTORC1*

In the presence of pro-growth endocrine signals, sufficient energy and chemical building blocks for macromolecular synthesis, mTORC1 activity shifts toward increased anabolism. These inputs are mainly dependent on diet. Therefore, mTORC1 is activated upon refeeding leading to the promotion of growth and energy storage in tissues. The opposite occurs during fasting to conserve limited nutrients and resources.

In the early 1990s, studies of rapamycin revealed that mTORC1 is a downstream mediator of several growth factor and mitogen dependent signaling pathways, which inhibit Tuberous Sclerosis Complex (TSC), a key negative regulator of mTORC1 signaling. Numerous growth factor pathways converge on TSC, including the insulin/insulin-like growth factor-1 (IGF-1) pathway, in which AKT phosphorylates and inhibits TSC2 (Inoki et al., 2002; Manning et al., 2002). TSC functions as a GTPase activating protein for the Rheb, a small GTPase (Inoki et al., 2003a; Tee et al., 2003). Rheb directly binds and activates mTORC1 (Long et al., 2005; Sancak et al., 2007).

Receptor tyrosine kinase-dependent Ras signaling also activates mTORC1 via the MAP Kinase ERK which also phosphorylates and inhibits TSC2 (Ma, L. et al., 2005; Roux et al., 2004). Other growth factor pathways upstream of TSC include WNT and  $\text{TNF}\alpha$ , an inflammatory cytokine, which both activate mTORC1 through inhibition of TSC1 (Inoki et al., 2006; Lee, D. F. et al., 2007). mTORC1 also responds to intracellular and environmental stresses that are unsuitable for cellular growth. For example, during glucose deprivation, the stress responsive metabolic regulator AMPK is activated and inhibits mTORC1. Inhibition in this pathway occurs both directly, through the phosphorylation of Raptor and indirectly, through phosphorylation of TSC2 (Gwinn et al.,

2008; Inoki et al., 2003b; Shaw et al., 2004). However, in cells lacking AMPK, glucose deprivation inhibits mTORC1 through inhibition of the Rag GTPases (Efeyan et al., 2013; Kalender et al., 2010). Hypoxia also inhibits mTORC1 in part through AMPK activation, and through the induction of REDD1 (Regulated in DNA damage and development 1). REDD1 also activates TSC (Brugarolas et al., 2004). The DNA damage-response pathway inhibits mTORC1 through the induction of p53 target genes which increases TSC activity (Feng et al., 2007).

Feeding leads to an increase in serum amino acid levels from the digestion of dietary proteins. mTORC1 activation is linked to changes in amino acid concentrations from the diet. Briefly, amino acid stimulation converts Rag GTPases to their active form, which they then bind to Raptor and recruit mTORC1 to close proximity of Rheb. In this pathway, mTORC1 signaling is only activate when both the Rags and Rheb are activated. mTORC1 senses both intra-lysosomal and cytosolic amino acids through distinct mechanisms. Several additional mechanisms by which amino acids regulate mTORC1 signaling have also been reported.

#### *1.2.4 mTORC1 Downstream Signaling*

As two well-characterized and critical downstream targets of mTORC1, ribosomal subunit S6kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP-1) mediate translation regulation and protein synthesis in cells upon phosphorylation by mTORC1 (Foster et al., 2010). mTORC1 also phosphorylates many metabolic enzymes to regulate cell growth and post-transcriptional regulation of lipid metabolism. In addition, mTORC1 stimulates cell growth by suppression of protein catabolism, through autophagy.

In absence of growth factors, unphosphorylated S6K1 and 4EBP-1 are associated with eukaryotic initiation factor (eIF)-3 complex and eIF4E, respectively. In response to growth factors, mTORC1 phosphorylates S6K and 4EBP-1. After phosphorylation by mTORC1 on Thr389, which dissociates S6K1 from eIF3, S6K1 then phosphorylates eukaryotic translation initiation factor 4B (eIF4B), a positive regulator of the 5' cap-binding eIF4F complex, this phosphorylation results in activation (Brunn et al., 1997; Gingras et al., 1999; Holz et al., 2005). S6K1 also phosphorylates programmed cell death protein 4 (PDCD4). This phosphorylation results in the degradation of PDCD4, an inhibitor of eIF4A (Dorrello et al., 2006). S6K1, through casein kinase 1 (CK1), leads to phosphorylation and activation of SRPK2. SRPK2 is a key RNA-binding regulator in mRNA processing. This downstream effect modifies splicing of pre-mRNAs that encoded lipogenic enzymes (Ma, J. et al., 2006). 4EBP-1 is phosphorylated by mTORC1 at multiple sites (Brunn et al., 1997; Gingras et al., 1999). After phosphorylation, 4EBP-1, unrelated to S6K1 signaling, is released from eIF4E (Hay et al., 2004). This inhibits translation by binding and sequestering of eIF4E to 4EBP-1, preventing formation of the eIF4F complex. Short term mTOR inhibition, as with rapamycin treatment, relatively suppressed general mRNA translation. However, the greatest effects were on mRNAs that contain pyrimidine-rich 5' TOP motifs. This comprises many genes involved in protein synthesis (Hsieh et al., 2012; Thoreen et al., 2012).

mTORC1 also phosphorylates many metabolic enzymes to regulate cell growth and post-transcriptional regulation of lipid metabolism (Lee, G. et al., 2017). Through an S6K1-dependent mechanism, mTORC1 promotes de novo lipid synthesis by activating



the sterol regulatory element-binding protein (SREBP) transcription factors, which control expression of fatty acid and cholesterol synthesis genes (Duvel et al., 2010; Porstmann et al., 2008). mTORC1 directly phosphorylates Lipin 1, which inhibits SREBP in the absence of mTORC1 signaling (Peterson, T. R. et al., 2011). In addition, mTORC1 activation of SREBP increases flux through the oxidative pentose phosphate pathway (PPP). This pathway uses carbons from glucose to produce intermediate metabolites needed for proliferation and growth. mTORC1 facilitates growth by altering glucose metabolism. This is done by inducing a shift from oxidative phosphorylation to glycolysis. mTORC1 also increases translation of HIF1a, a transcription factor that increases the expression of glycolytic enzymes, such as phosphofructo kinase (PFK) (Duvel et al., 2010).

In DNA replication and ribosome biogenesis for growing and proliferating cells, mTORC1 promotes the synthesis of nucleotides. mTORC1 increases ATF4-dependent expression of MTHFD2, a key protein that provides one-carbon units for purine synthesis (Ben-Sahra et al., 2016). Through phosphorylation of S6K1, mTORC1 activates carbamoyl-phosphate synthetase (CAD), which is a critical protein in de novo pyrimidine synthesis (Ben-Sahra et al., 2013; Robitaille et al., 2013). mTORC1 promotes cell growth by suppressing protein catabolism, mainly by autophagy. ULK1 is involved in the early step of autophagy. This kinase forms a complex with ATG13, FIP200, and ATG101, driving the formation of autophagosome. In absence of growth factors, mTORC1 phosphorylates ULK1, which prevents activation by AMPK (Kim, J. et al., 2011). mTORC1 also phosphorylates and inhibits the nuclear translocation of the transcription factor EB (TFEB). TFEB induces gene expression for lysosomal biogenesis and

autophagy processes (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). A second major way mTORC1 regulates protein turnover is through the ubiquitin-proteasome system (UPS). This pathway selectively targets proteins for degradation by the 20S proteasome following modification with ubiquitin. Inhibition of mTORC1 increases UPS through an increase in either protein ubiquitination, or through inhibition of ERK5, which increases the amount of proteasomal chaperones (Rousseau et al., 2016; Zhao et al., 2015). On the contrary, hyper activation of mTORC1 signaling can also lead to an increase in proteasome activity. This downstream signaling is mediated through NRF1, which increases the expression of proteasome subunits (Zhang, Y. et al., 2014).

#### *1.2.5 Upstream mTORC2*

In contrast to mTORC1, mTORC2 primarily functions as an effector of insulin/PI3K signaling. The mTORC2 subunit mSin1 contains a phosphoinositide-binding PH domain critical for insulin-dependent regulation of mTORC2 activity. In the absence of insulin, the PH domain of mSin1 inhibits mTORC2 catalytic activity (Liu, P. et al., 2015). mSin1 can also be phosphorylated by AKT, indicative of a positive-feedback loop where partial activation of AKT induces the activation of mTORC2, which mTORC2 then can phosphorylate leading to fully activated AKT (Yang, G. et al., 2015). In addition, another study showed that PI3K promotes localization of mTORC2 to ribosomes which activates mTORC2 kinase activity, although the mechanism remains unclear (Zinzalla et al., 2011). mTORC1 also regulates mTORC2 signaling due to the negative feedback loop between mTORC1 and insulin/PI3K signaling. mTORC1 downstream target, Grb10, is a negative regulator of insulin/IGF-1 receptor signaling residing upstream of mTORC2,

(Hsu et al., 2011; Yu et al., 2011). In addition, S6K1 suppresses mTORC2 activity through the phosphorylation-dependent degradation of insulin receptor substrate 1 (IRS1) (Harrington et al., 2004; Shah et al., 2004).

#### *1.2.6 mTORC2 Downstream Signaling*

While mTORC1 regulates cell growth and metabolism, mTORC2 controls proliferation and survival. mTORC2 phosphorylates several members of the AGC (PKA/PKG/PKC) family of protein kinase to control proliferation and survival. The first mTORC2 substrate to be identified was PKC $\alpha$ , a regulator of the actin cytoskeleton (Jacinto et al., 2004; Sarbassov et al., 2004). mTORC2 also phosphorylates several other members of the PKC family, including PKC $\delta$ , PKC $\zeta$ , and PKC $\gamma$  (Gan et al., 2012; Li, X. et al., 2014; Thomanetz et al., 2013). The PKC family regulate various aspects of cytoskeletal remodeling and cell migration. Serum and glucocorticoid inducible kinase (SGK1), a AGC-kinase that regulates ion transport as well as cell survival is another other well-known mTORC2 targets (Garcia-Martinez et al., 2008).

A critical role of mTORC2, however, is the phosphorylation and activation of AKT, a key effector of insulin/ PI3K signaling (Sarbassov et al., 2005). AKT is phosphorylated at two sites for complete activation. Phosphorylation at T308 by phosphoinositide-dependent kinase (PDK)-1 at the plasma membrane. and phosphorylation at S473 by mTORC2. Complete activation of AKT regulates processes like cellular growth, and apoptosis through the phosphorylation and subsequent inhibition of several key substrates. These substrates include the FoxO1/3 $\alpha$  transcription factors, the metabolic regulator GSK3 $\beta$ , and the mTORC1 inhibitor TSC2. In glucose metabolism, phosphorylated AKT promotes glucose transporter type (GLUT)-4 translocation to the plasma membrane, allowing

glucose uptake in response to insulin stimulation (Kohn et al., 1996; Manning et al., 2007). In addition, activated AKT regulates *de novo* fatty acid synthesis through activation of ATP-Citrate Lyase (ACL) (Berwick et al., 2002).

### **1.3 Lipid Metabolism**

#### *1.3.1 Lipoproteins and Lipid Transport*

##### **Exogenous Lipoprotein Pathway**

The exogenous lipoprotein pathway starts with the incorporation of dietary lipids into chylomicrons (CM) in the intestine. In the fed state, the absorbed fatty acids and monoacylglycerols are utilized to synthesize triglycerides. The key enzymes required for triglyceride synthesis are monoacylglycerol acyltransferase (MGAT) and diacylglycerol transferase (DGAT). MGAT catalyzes the addition of a fatty acid to monoacylglycerol while DGAT catalyzes the addition of a fatty acid to diacylglycerol resulting in triglyceride formation. The triglycerides and cholesterol esters are packaged into chylomicrons in the endoplasmic reticulum. The formation of chylomicrons in the endoplasmic reticulum requires the synthesis of Apo B-48 by the intestinal cell. Microsomal triglyceride transfer protein (MTP) is required for the movement of lipid from the endoplasmic reticulum to the Apo B-48. The absence of MTP results in the inability to form chylomicrons (Abetalipoproteinemia) (Dallinga-Thie et al., 2010). Chylomicrons are secreted to the systemic circulation and not delivered directly to the liver via the portal circulation. This facilitates the delivery of the nutrients contained in the chylomicrons to adipose tissue. In the circulation the triglycerides carried in chylomicrons are metabolized in adipose tissue by lipoprotein lipase (LPL) and chylomicron remnants are formed. The chylomicron

remnants are then taken up by the liver. Chylomicrons (CM) are large triglyceride rich particles made by the intestine, which are involved in the transport of dietary triglycerides and cholesterol to peripheral tissues and liver. Apo B-48 is the core structural protein and each chylomicron particle contains one Apo B-48 molecule. The size of chylomicrons varies depending on the amount of fat ingested. A high fat meal leads to the formation of large chylomicron particles due to the increased amount of triglyceride being transported. The exogenous lipoprotein pathway results in the efficient transfer of dietary fatty acids to adipose tissue for energy utilization and storage. The cholesterol is delivered to the liver where it can be utilized for the formation of VLDL, bile acids, or secreted back to the intestine via secretion into the bile. In normal individuals this pathway can handle large amounts of fat (100 grams or more per day) without resulting in marked increases in plasma triglyceride levels. In fact, in a normal individual, a meal containing 75 grams of fat results in only a very modest increase in postprandial triglyceride levels.

#### The Endogenous Lipoprotein Pathway

The endogenous lipoprotein pathway begins in the liver with the formation of very low density lipoproteins (VLDL). VLDL are triglyceride rich. They contain apolipoprotein B-100, C-I, C-II, C-III, and E. Apo B-100 is the core structural protein and each VLDL particle contains one Apo B-100 molecule. Similar to chylomicrons the size of the VLDL particles can vary depending on the quantity of triglyceride carried in the particle. When triglyceride production in the liver is increased, the secreted VLDL particles are large. In the fasted state, liver triglycerides and cholesterol esters are transferred in the endoplasmic reticulum to newly synthesized Apo B-100. Similar to the intestine this

transfer is mediated by MTP. The availability of triglycerides is the primary determinant of the rate of VLDL ApoB-100 synthesis. If the supply of triglyceride is limited the newly synthesized Apo B is rapidly degraded. Thus, in contrast to many proteins the rate of synthesis of the Apo B-100 is not the major determinant of the rate of secretion. Rather the amount of lipid available determines whether Apo B-100 is degraded or secreted. MTP is required for the early addition of lipid to Apo B-100 particles but additional lipid is added via pathways that do not require MTP. Loss of function mutations in either Apo B-100 or MTP result in the failure to produce VLDL and marked decreases in plasma triglyceride and cholesterol levels (Familial hypobetalipoproteinemia or abetalipoproteinemia) (Tiwari et al., 2012). VLDL particles are transported to peripheral tissues where the triglycerides are hydrolyzed by LPL and fatty acids are released. This process is very similar to that for chylomicrons and there is competition between the metabolism of chylomicrons and VLDL. High levels of chylomicrons can inhibit the clearance of VLDL. The removal of triglycerides from VLDL results in the formation of VLDL remnants (Intermediate density lipoproteins (IDL)). In a pathway analogous to the removal of chylomicron remnants these IDL particles can be removed from the circulation by the liver via binding of Apo E to LDL and LRP receptors. However, while the vast majority of chylomicron remnants are rapidly cleared from the circulation by the liver, only a fraction of IDL particles are cleared (approximately 50% but varies). The remaining triglycerides in the IDL particles are hydrolyzed by hepatic lipase leading to a further decrease in triglyceride content and the exchangeable apolipoproteins are transferred from the IDL particles to other lipoproteins leading to the formation of LDL, which are taken up by via the LDL receptor in numerous tissues including the liver,

which is the predominant site of uptake. These LDL particles predominantly contain cholesterol esters and Apo B-100. Thus, LDL is a product of VLDL metabolism.

### Lipoprotein Lipase

In muscle and adipose tissue lipoprotein lipase (LPL) is expressed at high levels. LPL belongs to a family of lipases, which includes hepatic lipase, and endothelial lipase, and hydrolyzes triglycerides in chylomicrons and very low-density lipoproteins (VLDL). The hydrolyzed triglycerides yields two free fatty acids and one monoacylglycerol (MG) (Mead et al., 2002; Rinninger et al., 1998). LPL is composed of two distinct regions. The active site of LPL is in the N-terminal domain (Mead et al., 2002; Rinninger et al., 1998; Wang, C. S. et al., 1992). The C-terminal domain appears to confer LPL's substrate specificity; it has a higher affinity for large triacylglyceride-rich lipoproteins than cholesterol-rich lipoproteins (Lookene et al., 2000). Lipids interact with the active site (Mead et al., 2002). The gene that encodes lipoprotein lipase, which is expressed in the heart, muscle, and adipose tissue. LPL is controlled transcriptionally and post transcriptionally (Wang, H. et al., 2009). LPL isozymes are differentially regulated depending on the tissue. In adipocytes, insulin activates LPL and its secretion to the capillary endothelium. In muscle, insulin decreases expression of LPL (Kiens et al., 1989). In muscle and heart, LPL is activated by glucagon and adrenaline (Braun et al., 1992; Mead et al., 2002).

LPL is synthesized in skeletal and cardiac muscle in the fasted state and in adipocytes in fed state. After translation, the newly synthesized protein is glycosylated in the endoplasmic reticulum. Homodimerization occurs prior to secretion from cells (Ong et al., 1989; Vannier et al., 1989). LPL is then secreted as a homodimer and

translocated across endothelial cells to the capillary lumen by the protein glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (Beigneux et al., 2007; Davies et al., 2010; Mead et al., 2002). A conformational change occurs in order for LPL to form homodimers and become catalytically active (Braun & Severson, 1992; Semb et al., 1989; Wong et al., 1994). Lipase maturation factor 1 (LMF1) plays a key role in the stabilization and movement of LPL from muscle cells and adipocytes to the capillary endothelial cell surface. Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1) anchors LPL to the capillary endothelium.

The concentration of LPL on the surface of endothelial cells is regulated by managing the flux of secreted LPL and by the activity of LPL present on the endothelium. A key protein involved in controlling the activity of LPL is ANGPTL4, a local inhibitor of LPL. Expression of ANGPTL4 accounts for the inhibition of LPL activity in white adipose tissue during fasting (Dijk et al., 2014; Zhang, R., 2016). Feeding induces the ANGPTL8–ANGPTL3 pathway to inhibit LPL in cardiac and skeletal muscles, directing circulating triglycerides for uptake by white adipose tissue. The reverse is true during fasting, directing triglycerides to muscles (Zhang, R., 2016).

Activation of LPL by Apo C-II, carried on the chylomicrons, leads to the hydrolysis of the triglycerides that are carried in the chylomicrons resulting in the formation of free fatty acids, which can be taken up by the adjacent adipocytes for either energy production or storage. Fatty acid transport proteins (FATPs) and CD36 facilitate the uptake of fatty acids into adipocytes and muscle cells. Some of the free fatty acids released from chylomicrons bind to albumin and can be transported to other tissues.



The glycerol backbone of the lipid is then able to enter the active site and is hydrolyzed. it is believed that release of product into circulation is the rate-limiting step in the reaction (Wang, C. S. et al., 1992).

Loss of function mutations in LPL, Apo C-II, GPIHPB1, lipase maturation factor 1, and Apo A-V can result in marked hypertriglyceridemia (chylomicronemia). In addition, there are proteins that inhibit LPL activity. Apo C-III inhibits LPL activity and loss of function mutations in this gene are associated with increases in LPL activity and decreases in plasma triglyceride levels. Similarly, angiopoietin like protein 3 and 4, which target LPL for inactivation, regulate LPL activity. Loss of function mutations in these proteins also are associated with decreases in plasma triglyceride levels. In mice, overexpression of LPL has been shown to cause insulin resistance, and to promote obesity (Delezie et al., 2012; Ferreira et al., 2001; Kim, J. K. et al., 2001).

### *1.3.2 Lipolysis*

Lipolysis is the process by which triglycerides (TG) are hydrolyzed to free fatty acids (FFA) and glycerol. In adipocytes, this is achieved by sequential action of adipose TG lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerols (MG) lipase. The activity in the lipolytic pathway is tightly regulated by hormonal and nutritional factors. Under conditions of negative energy balance such as fasting and exercise, stimulation of lipolysis results in a profound increase in FFA release from adipose tissue (AT). This response is crucial in order to provide the organism with a sufficient supply of substrate for oxidative metabolism. However, failure to efficiently suppress lipolysis when FFA demands are low can have serious metabolic consequences and is believed to be a key mechanism in the development of type 2 diabetes in obesity.

The primary role of adipose tissue is to release stored fatty acids during times of increased energy demand, such as exercise or fasting. Lipolysis involves the sequential hydrolysis of triacylglycerols (TG) by specific enzymes and results in the liberation of a fatty acid at each step with the generation of diacylglycerols (DG), monoacylglycerols (MG) and glycerol. This process is controlled by three major lipases; adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerols lipase (MAGL) (Tsiloulis et al., 2015). Lipolysis is tightly regulated by a number of pro-lipolytic and anti-lipolytic (e.g. insulin) hormones. Upon  $\beta$ -adrenergic activation, a cascade of signaling events results in maximal ATGL and HSL activity via phosphorylation events, protein–protein interactions and accumulation on the surface of lipid droplets, facilitating direct interaction between the lipases and TG (Arner et al., 2014; Tsoli et al., 2016). Lipolysis is suppressed by insulin via mechanisms that antagonize the  $\beta$ -adrenergic pathway (Morigny et al., 2016).

Three independent laboratories identified ATGL as a major triglyceride lipase in 2004 (Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004). ATGL is the rate-limiting enzyme in the lipolytic cascade, exhibiting high substrate specificity for TG, and very weak activity against DG (Eichmann et al., 2012; Zimmermann et al., 2004). Pioneering studies in ATGL<sup>−/−</sup> mice demonstrated reduced basal and catecholamine-stimulated lipolysis and increased adipocyte mass compared with control mice, clearly demonstrating that ATGL is a crucial lipase in the lipolytic chain. Phosphorylation at ATGL Ser406 (Ser404 in humans) increases lipolysis in adipocytes and there is no known functional role for the other phosphorylation sites (Haemmerle et al., 2006).

Phosphorylation of ATGL at Thr372 is required for ATGL to localize to the lipid droplet for efficient lipolysis, although the activating kinase is unknown (Xie et al., 2014).

PLIN1 is a lipid droplet associated protein which acts as a 'scaffold' by facilitating access of lipases to the lipid droplet and mediating protein–protein interactions in adipocytes. Its most prominent role is to reduce lipolysis under 'basal' or 'spontaneous' conditions.

PLIN1 binds CGI-58 with high affinity and suppresses the interaction of CGI-58 and ATGL, thereby suppressing lipolysis and facilitating TG accumulation (Granneman et al., 2009). PLIN1 phosphorylation by PKA is necessary for maximal lipolysis (Brasaemle et al., 2009). Key phosphorylation sites maximize the activation of ATGL-dependent lipolysis, and the fragmentation and dispersion of lipid droplets to increase the surface area for lipase binding in mice, and are conserved in humans, suggesting similar regulation (Marcinkiewicz et al., 2006; McDonough et al., 2013). Germline ablation of *Plin1* result in animals with increased basal lipolysis and decreased stimulated lipolysis (Tansey et al., 2001).

### 1.3.3 Lipogenesis

*De novo* sterol and fatty acid synthesis are regulated by signaling pathways, mainly the insulin signaling pathway. Both hepatocytes and adipocytes synthesize significant amounts of lipid *de novo*. This fatty acid biosynthesis pathway involves cytosolic acetyl-CoA, derived from glucose or amino acid catabolism, to form the hydrophobic carbon backbone of lipids through acetyl-CoA carboxylase. This process comprises many steps requiring many specific enzymes. The most important protein is the SREBPs, transcription factors that stimulate the expression of genes encoding lipogenic enzymes (Horton, 2002). The three SREBP isoforms are encoded by two genes. Under conditions

of abundant nutrients, full-length SREBP is retained in the endoplasmic reticulum, preventing cleavage into the isoforms (Jeon et al., 2012). Depletion of intracellular nutrients results in transport to the Golgi, in cleavage release the active SREBP, which then enters the nucleus and induces transcription. SREBP1a and 1c are primarily implicated in the control of genes involved in fatty acid synthesis (Shimano et al., 1997). SREBP2 plays a role in the transcription of steroidogenic and cholesterol genes (Horton et al., 1998; Horton et al., 2003; Linden et al., 2018; Rong et al., 2017). Although the different SREBP isoforms activate transcription of different sets of genes, there is overlap between the targets of the different isoforms. The tissue specificity of these preferences, which has not been fully established. Lipin-1 is a phosphatidic acid phosphatase required for glycerolipid biosynthesis. This protein also serves as a transcriptional coactivator that regulates the expression of lipogenic genes (Csaki et al., 2010).

#### *1.3.4 Adipogenesis and Adipose Tissue*

White adipose tissue is known as an organ that regulates systemic lipid homeostasis by storing and releasing free fatty acids (Rutkowski et al., 2015; Tran, T. T. et al., 2010). Adipose tissue is essential for normal health. Multiple metabolic defects that occur in patients with lipodystrophies, are characterized by decreased adipose tissue (Huang-Doran et al., 2010; Qiang et al., 2016). In contrast, the excessive accumulation of fat in the form of triglycerides is associated with metabolic dysfunctions. These dysfunctions can cause insulin resistance and type 2 diabetes, cardiovascular disease, and some cancers (Bjorndal et al., 2011; Despres, 2006; Tran, T. T. & Kahn, 2010).

Adipose tissue is mainly categorized as white (WAT) or brown (BAT) based on several criteria. This includes intracellular lipid droplet morphology, mitochondrial number and metabolic functions (fat storage vs fat catabolism). There is a third type of adipocyte, the 'beige' adipocyte, which stems from white adipocytes upon cold exposure or other stimuli. 'Beige' adipose shares structural and functional features of both WAT and BAT (Kajimura et al., 2010). WAT is most abundant adipose depot, comprising 5-60% of body mass (Lee, M. J. et al., 2013). Adipose tissue is mostly located in subcutaneous locations (i.e. under the skin) (Park et al., 2014). Subcutaneous adipose tissue comprises up to ~80% of the whole-body adipose mass. The omental, retroperitoneal, mesenteric and perigonadal depots make up the majority of the visceral adipose tissue, which is located within the intra-abdominal regions and contributes to ~5– 20% of body mass (Lee, M. J. et al., 2013).

Adipogenesis, a complex developmental process, involves commitment of multipotent stem cells to adipose lineage and the accumulating lipids to form mature adipocytes (Cawthorn et al., 2012). Several transcription factors regulate the differentiation of preadipocytes into mature adipocytes. These transcription factors include the CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ), which is a nuclear receptor needed for the whole adipogenic program. When preadipocytes are stimulated to differentiate, expression of C/EBP $\delta$  and C/EBP $\beta$  is activated. This prompts the induction of C/EBP $\alpha$  and the expression of PPAR $\gamma$ , which directly promotes the expression of genes involved in fatty acid metabolism, triglyceride metabolism, and lipid storage.

## 1.4 mTORC 1 and 2 in Lipid Metabolism

The transition between fasting and feeding is accompanied by profound alterations in circulating levels of nutrients and growth factors. These changes modulate whether anabolic or catabolic processes are turned on in tissues. Postprandial metabolism, the period during or after refeeding, is very dynamic and allows tissues to efficiently use and store the ingested nutrients to support anabolism. Cells require an ample amount of lipids to provide support for energy metabolism and membrane biogenesis. Lipogenesis is a cellular process by which acetyl-CoA is converted to fatty acids. This important anabolic process occurs in several tissues following refeeding and is dependent on circulating insulin levels (Kersten, 2001). mTOR activation, in addition to promoting anabolism, also blocks several catabolic processes. In the fasting state, levels of nutrients and growth factors are limited. Adipose tissue lipolysis, the breakdown of intracellular triglycerides, provides fuel for utilization by other tissues in need of nutrients (Zechner et al., 2012). This process is inhibited by both mTOR complexes when excess nutrients are readily available. Since mTOR signaling pathways responds to nutrient and growth factor levels, the roles of both complexes in regulating lipogenesis and lipolysis has been extensively studied over the past few years.

### *1.4.1 mTOR Complexes in Lipogenesis*

#### mTORC1

mTORC1 plays a central role in promoting lipid biogenesis by regulating the expression of many lipogenic genes, most importantly sterol regulatory element-binding proteins (SREBPs), transcription factors involved in lipid synthesis (Laplane et al., 2013).

mTORC1 positively regulates the activation of SREBPs at multiple levels, which includes processing, trafficking, and transcription (Bakan et al., 2012). In 2008, it was discovered that rapamycin prevents the nuclear accumulation of mSREBP1, normally mediated through AKT, and the subsequent induction of lipogenic genes (Porstmann et al., 2008). Previous studies have also reported that rapamycin reduces the expression of several lipogenic genes (Mauvoisin et al., 2007; Peng, T. et al., 2002). Deletion of *Raptor*, but not *Rictor*, prevents SREBP1 processing and decreases the expression of lipogenic genes (Porstmann et al., 2008). This was also observed *in vivo*. Mice treated with rapamycin had reduced hepatic SREBP1 processing and decreased lipogenic gene expression (Yecies et al., 2011). S6K1 has also been proven to regulate SREBPs processing downstream of mTORC1 signaling (Duvel et al., 2010; Li, S. et al., 2010; Liu, X. et al., 2012; Owen et al., 2012; Wang, B. T. et al., 2011). Genetic deletion of the TSC complex *in vitro*, which leads to increased mTORC1 signaling, stimulated nuclear accumulation of mSREBP1 and de novo lipogenesis (Duvel et al., 2010). In liver-specific *Tsc1* null mice, constitutive mTORC1 activation worsened the feedback inhibition of insulin signaling, which also worsened AKT activity. Decreased AKT signaling correlated with increased expression of *Insig2a*, an inhibitor of SREBP1 (Yecies et al., 2011). An increase in insulin also induces expression of *Srebf1* in the liver (Foretz et al., 1999; Horton et al., 1998; Shimomura et al., 1999). This process is inhibited with both pharmacological and genetic inhibition of mTORC1 (Duvel et al., 2010; Li, S. et al., 2010; Owen et al., 2012; Porstmann et al., 2008; Titchenell et al., 2016; Wan et al., 2011; Yecies et al., 2011). mTORC1 also regulates SREBPs through phosphorylation of Lipin-1, which induces the nuclear accumulation of SREBPs (Peterson, T. R. et al., 2011). These studies demonstrate the importance of mTORC1 in regulating lipogenesis.

## mTORC2

mTORC2 also plays a critical role in modulating lipid synthesis. Deletion of *Rictor* in the liver decreases SREBP activity, the expression of lipogenic genes, and lipid accumulation in the liver (Hagiwara et al., 2012; Yuan et al., 2012). Deletion of *Rictor* in brown adipose tissue lead to a decrease in lipogenesis (Hung et al., 2014). Proposed mechanism of action differs between. In the liver-specific *Rictor* KO mice, overexpression of a constitutively activated AKT isoform rescued the defects in lipogenesis (Hagiwara et al., 2012). Loss of mTORC2-mediated AKT phosphorylation on Serine 473 partially reduces AKT function, which impair SREBP1 activation AKT has been shown to control lipogenesis through its action on SREBP1, through *Insig2a*, direct phosphorylation SREBP1, and by blocking glycogen synthase kinase 3-mediated degradation of mSREBP1 (Bengoechea-Alonso et al., 2009; Sundqvist et al., 2005; Yabe et al., 2003; Yecies et al., 2011; Yellaturu et al., 2009).

### 1.4.2 mTOR Complexes in Lipolysis

Rapamycin treatment *in vivo* and *in vitro* has been shown to stimulate adipocyte triglyceride hydrolysis and free fatty acid release (Aggarwal et al., 2006; Chakrabarti et al., 2010; Houde et al., 2010; Lopes et al., 2014; Morrisett et al., 2003; Pereira et al., 2013; Soliman et al., 2010). Several studies suggest that mTORC1 can affect lipolysis, however, activation of lipolysis observed with rapamycin can also be due to the indirect inhibition of the mTORC2 (Chakrabarti et al., 2010; Chakrabarti et al., 2013). Additional studies are needed to better understand the mechanisms of action and the contribution of each mTOR complex to the regulation of lipolysis.



## mTORC1

Various experimental conditions link mTORC1 and adipose tissue. Humans and animal treated with rapamycin had increased plasma free fatty acids (Aggarwal et al., 2006; Houde et al., 2010; Morrisett et al., 2003; Soliman et al., 2010). Similarly, isolated adipocytes treated with rapamycin increased *ex vivo* basal and stimulated lipolysis (Chakrabarti et al., 2010; Lopes et al., 2014; Pereira et al., 2013). In differentiated 3T3-L1 cells, rapamycin treatment or *Raptor* deletion had the same effect (Chakrabarti et al., 2010; Soliman et al., 2010). Overexpression of Rheb, which leads to activation of mTORC1, inhibited lipolysis in 3T3-L1 cells (Chakrabarti et al., 2010). In isolated adipocytes, loss of S6K1 induced lipolysis and loss of 4E-BP1 reduced this effect (Le Bacquer et al., 2007; Um et al., 2004). Results from these genetic studies suggest a direct role for mTORC1 signaling in the control of triglyceride break down and fatty acid release from adipocytes. However, one study using mice with adipose-specific deletion of *Raptor*, did not observe any effect on lipolysis (Polak et al., 2008). Although the reason for this inconsistency is unknown, this study used mice expressing cre recombinase under the promoter of the fatty acid binding protein 4 (*Fabp4*) gene which encodes adipocyte protein 2 (aP2), which has been shown to not be adipocyte specific (Graves et al., 1992; Lee, K. Y. et al., 2013; Mullican et al., 2013; Shan et al., 2013; Urs et al., 2006).

It has been suggested that mTORC1 affects the lipolysis through expression of adipose triglyceride lipase (ATGL), the rate limiting enzyme that initiates the first step of lipolysis, converting triglycerides to diacylglycerols (Zechner et al., 2012). Overexpression of mTORC1 signaling has been shown to reduce expression of *Atgl* (Chakrabarti et al.,

2010). *In vitro* and *in vivo* studies demonstrated that inhibition of mTORC1 by rapamycin increases *Atgl* expression. It has been proposed that mTORC1, upon activation by insulin or growth factors, regulates *Atgl* expression by increasing levels of transcription factor early growth response-1 (EGR1) (Chakrabarti et al., 2013). Although the mechanism of how mTORC1 increases EGR1 is unknown, EGR1 has been shown to inhibit expression of *Atgl* by binding directly to the promoter. Upon refeeding, decreases in plasma free fatty acids occur within one hour, suggesting that the regulation of triglyceride hydrolysis occur through posttranslational modifications, not through changes in lipase expression (Hernandez et al., 2010; Laplante et al., 2003; Zechner et al., 2012). Therefore, mTORC1 transcriptional regulation of *Egr1* and *Atgl* most likely does not play a role in the inhibition of lipolysis upon refeeding.

Independent of changes in ATGL, inhibition of mTORC1 by rapamycin has been shown to increase lipolysis, by stimulating protein kinase A (PKA)-mediated phosphorylation of hormone-sensitive lipase on Ser563 (Soliman et al., 2010). Phosphorylation of this site is necessary for complete hydrolysis of triglycerides (Shen et al., 1998). This study reported that the inhibition of mTORC1 increased catecholamine-stimulated lipolysis, which was independent of changes in the levels of intracellular cAMP and phosphorylation of PKA.

## mTORC2

mTORC2 has also been shown to negatively regulate adipose tissue lipolysis. Adipose-specific KO of *Rictor* decreases the ability of insulin to suppress lipolysis of adipocytes (Kumar et al., 2010). These adipose-specific *Rictor* KO mice displayed elevated plasma

glycerol and free fatty acid levels, ectopic fat deposition, and developed insulin resistance. This higher lipolytic rate was linked to reduced phosphorylation of AKT, increased activity of PKA, and increased higher phosphorylation of HSL on Ser563. These results suggest that mTORC2 mediates triglyceride breakdown in fat cells through the antilipolytic functions of AKT. However, not all studies on adipose-specific *Rictor* KO mice yielded the same results. One study did not observe any changes in plasma free fatty acids with loss of mTORC2 (Cybulski et al., 2009). The reason for this discrepancy is not clear but could be due to differences in experimental procedures.

#### 1.4.3 mTOR Complexes in Adipogenesis

##### mTORC1

Many studies support a role for mTORC1 in the regulation of adipogenesis. These studies, using rapamycin, reported that rapamycin abrogates adipocyte differentiation in various cell lines *in vitro* (Bell et al., 2000; Cho et al., 2004; El-Chaar et al., 2004; Gagnon et al., 2001; Kim, J. E. et al., 2004; Polak et al., 2008; Yeh et al., 1995; Zhang, H. H. et al., 2009). Because prolonged treatment with rapamycin can sometimes inhibit mTORC2, specific contribution of mTORC1 in adipogenesis requires genetic inhibition of mTORC1 (Lamming et al., 2012; Sarbassov et al., 2006). Polak et al. knocked down *Raptor*, the essential mTORC1 component, in 3T3-L1 preadipocytes (Polak et al., 2008). In this model, loss of mTORC1 signaling drastically impaired adipogenesis. A similar result was observed when mouse embryonic fibroblasts (MEFs) with deletion of *Raptor* were differentiated *in vitro* (Polak et al., 2008). MEFs with constitutively activate mTORC1 by deletion of *Tsc2*, yielded the opposite result, with increased adipogenesis (Zhang, H. H. et al., 2009). The regulation of mTORC1 on adipogenesis has also been

proven in animal models. Several groups have shown that rapamycin treatment in rodents decreases adipose tissue (Chang et al., 2009a; Chang et al., 2009b; Houde et al., 2010). Using the Cre-Lox approach on mice with the *Raptor* gene floxed, *in vivo* effects of mTORC1 in adipose tissue regulation can be studied. Initial *in vivo* studies utilized the *aP2* promoter, however this targets not only the adipose but other tissues (Lee, K. Y. et al., 2013; Martens et al., 2010). Nevertheless, these mice had impaired lipid accumulation in adipose tissue and were protected from diet induced obesity (Polak et al., 2008). It was originally reported that rapamycin blocks the clonal expansion of preadipocytes into mature adipocytes, thus impairing adipogenesis (Yeh et al., 1995). Later, other groups studied the effect of rapamycin following induction of adipocyte differentiation. Rapamycin treatment still impaired adipogenesis indicating that mTORC1 regulates adipose terminal differentiation, most likely through a mechanism independent of the effects on proliferation (Bell et al., 2000; Cho et al., 2004; Gagnon et al., 2001). These findings support another group's results in which rapamycin inhibits the expression of C/EBPs and PPAR $\gamma$ , co-regulators of transcriptional activity in adipose differentiation. In addition, the two main downstream mTORC1 targets, S6K1 and 4E-BPs have also been shown to regulate adipocyte formation. Mice null for *S6K1* were not able to increase adipocyte cell number, and had decreased expression of early adipogenic transcription factors, C/EBPs (Carnevali et al., 2010). In addition, KD of S6K1 or inhibition with PF4708671 prevented preadipocytes differentiation *in vitro* (Yoon, M. S. et al., 2013). However, over expression of S6K1 was unable to rescue rapamycin's suppression of adipogenesis (Kim, J. E. & Chen, 2004). MEFs with loss of 4E-BP1/2 (similar state to constitutive active mTORC1), had increased expression of C/EBP $\alpha$  and PPAR $\gamma$  *in vitro* (Le Bacquer et al., 2007).

## mTORC2

In response to insulin, AKT stimulates glucose uptake and activates adipogenesis by promoting the activation of PPAR $\gamma$  (Kohn et al., 1996; Magun et al., 1996; Menghini et al., 2005; Nakae et al., 2003; Peng, X. D. et al., 2003; Rosen et al., 2006; Shearin et al., 2016). Therefore, it was first assumed that mTORC2 would play a key role in regulating adipogenesis. However, adipose-specific *Rictor*, the essential component of mTORC2, KO mice did not display any defect in adipose proliferation or accumulation (Cybulski et al., 2009; Kumar et al., 2008). Thus, it was concluded that mTORC2 is non-essential for adipogenesis *in vivo*, more specifically, terminal differentiation and adipocyte maintenance. In order to study the role of mTORC2 in the initial phases of adipocyte development, one group used *Rictor* floxed mice with *myogenic factor 5* (*Myf5*)-Cre recombinase, which will delete *Rictor* from white adipocyte progenitors (Hung et al., 2014). These mice accrued less adipose mass. Reports from two other studies, utilizing deletion of *Rictor* in MEFs or adipocyte precursors also impaired early adipogenesis (Hung et al., 2014; Yao et al., 2013).

### 1.5 Goal of this work

Aging is associated with a gradual decline in physiological functions of tissues (Sabatini, 2017). The process of aging in certain tissues can lead to a variety of disorders, this includes neurodegeneration, obesity, diabetes, and cardiovascular (Vezina et al., 1975). mTOR, which plays central role in age-related processes, represents an appealing target to ameliorate age-related pathologies. Rapamycin, an inhibitor of mTOR signaling, is the main pharmacological agent that can prolong lifespan in several model organisms (Baker et al., 1978).

Despite the studies that support the role of rapamycin or rapalogs to expand life span, the function as an immunosuppressant might be of concern. A decline in immune function leads to infection-related morbidity and mortality in the elderly (Murphy, 2010; Saxton & Sabatini, 2017). Another limitation of rapamycin is the inhibition of mTORC2 after chronic exposure in mice (Lamming et al., 2012; Sarbassov et al., 2006). In addition to rapamycin, active-site mTOR inhibitors also inhibit mTORC2 (Bhat et al., 2015). Inhibition of mTORC2 reduces life span and is associated with adverse changes in metabolism, such as insulin resistance, which negatively impacts life span (Lamming, 2014; Soukas et al., 2009). Therefore, much effort is being invested in developing specific inhibitors that suppress all mTORC1 signaling, including phosphorylation of 4E-BP1, but do not effect on mTORC2 signaling,

There is mounting evidence suggesting that both mTOR complexes are involved in lipid mobilization and transport. Metabolic dysfunction, such as obesity, induces chronically elevated mTORC1 signaling in liver and adipose, further contributing to the development of hypertriglyceridemia, resulting from the hyperglycemia and hyperinsulinemia associated with obesity (Haas, J. T. et al., 2012; Haas, M. E. et al., 2013; Khamzina et al., 2005; Um et al., 2004). In type 2 diabetes and insulin resistance, mTORC1 signaling remains active most likely due to persistent hyperinsulinemia, leading to increased de novo lipogenesis. Concurrently, insulin resistance in adipose also stimulates a steady increase in lipolysis, causing an increase in NEFA in the circulation. NEFA are mainly delivered to the liver but in excess, these lipids can accumulate in organs such as the skeletal muscle, pancreas, and heart, commonly referred to as ectopic fat deposition (Haas, M. E. et al., 2013; Hocking et al., 2013; Snel et al., 2012). The central role of

mTORC1 in regulating lipid homeostasis and its persistent activation in obesity and type 2 diabetes suggests mTORC1 inhibitors would offer therapeutic benefits in metabolic diseases. However, patients treated with mTORC1 inhibitors experience increased insulin resistance, hypertriglyceridemia, hypercholesterolemia, and elevated plasma FFA (Kasiske et al., 2008). Accordingly, plasma levels of both ApoB100 and ApoCIII were increased in rapamycin treated patients, indicating elevated circulating VLDL (Aggarwal et al., 2006; Morrisett et al., 2002). However, these studies only examined the effects of lipids in the fasted state. Lipid homeostasis requires a balance in lipid storage and synthesis in the fed state as well. Despite that mTOR inhibition clearly extends life span, the negative side effects associated with direct inhibition of mTOR complexes provide support for the identification of potential bioactive and therapeutic compounds capable of mitigating the adverse metabolic side effects. However, there are still outstanding questions regarding the negative metabolic effects and much work is still needed to fill the gaps in knowledge related to the function of mTOR in the context of lipid homeostasis. This work fills in some of those gaps in knowledge

In the experiments described below, we asked whether mTORC1 and mTORC2 signaling in adipocyte influences whole body lipid homeostasis, and if inhibition of these complexes induces hyperlipidemia. We also asked whether adipose tissue mediates the adverse lipid effects of rapamycin treatment. In addition, answers to these questions will fill in the gaps of knowledge that remain regarding the roles of mTOR in adipose tissue. We show that loss of either mTORC1 or mTORC2 results in hyperlipidemia, suggesting that mTOR is required *in vivo* for adipocyte maintenance of lipid homeostasis. We show that enhanced lipolysis upon refeeding increases plasma triglyceride levels in the

context of rapamycin treatment and that both complexes are involved in regulating this lipolytic process. However, mTORC1, not mTORC2, is required for proper adipocyte lipolysis to maintain circulating plasma lipid levels. Additionally, we provide evidence that loss of adipocyte mTOR signaling is not solely responsible for the rapamycin induced hyperlipidemia.



## **CHAPTER 2: mTORC1 restrains adipocyte lipolysis to prevent systemic hyperlipidemia (Paolella et al., 2020)**

## 2.1 Abstract

Pharmacological agents targeting the mTOR complexes are used clinically as immunosuppressants and anticancer agents, and can extend lifespan in model organisms. An undesirable side effect of these drugs is hyperlipidemia. Despite multiple roles that have been described for mTOR complex 1 (mTORC1) in lipid metabolism, the etiology of hyperlipidemia remains incompletely understood. The objective of this study was to determine the influence of adipocyte mTORC1 signaling in systemic lipid homeostasis *in vivo*. We characterized systemic lipid metabolism in mice lacking the mTORC1 subunit raptor (Raptor<sup>aKO</sup>), the key lipolytic enzyme ATGL (ATGL<sup>aKO</sup>), or both (ATGL-Raptor<sup>aKO</sup>) in adipocytes. Mice lacking mTORC1 activity in adipocytes failed to completely suppress lipolysis in the fed state and displayed prominent hypertriglyceridemia and hypercholesterolemia. Blocking lipolysis in adipose tissue restored normal levels of triglycerides and cholesterol in the fed state, as well as the ability to clear triglycerides in an oral fat tolerance test. Unsuppressed adipose lipolysis in the fed state interferes with triglyceride clearance and contributes to hyperlipidemia. Adipose tissue mTORC1 activity is necessary for appropriate suppression of lipolysis and for the maintenance of systemic lipid homeostasis.

## 2.2 Introduction

Therapeutic inhibition of mechanistic Target Of Rapamycin (mTOR) is important for immunosuppression and for the treatment of certain cancers (Benjamin et al., 2011). Recently, interest in this pathway has been heightened by the demonstration that the prototypical mTOR inhibitor, rapamycin, extends lifespan in model organisms from yeast to rodents (Harrison et al., 2009; Medvedik et al., 2007; Miller et al., 2011). However, these studies have also served to highlight our incomplete understanding of the mechanisms by which mTOR signaling influences physiology in different cell types, and of the etiology of the undesirable side effects that can accompany mTOR inhibition, which include an increased risk of new-onset diabetes and dyslipidemia, a major risk factor for cardiovascular diseases (Morrisett et al., 2003; Parekh et al., 2012). mTOR is a serine/threonine protein kinase that nucleates two structurally and functionally distinct complexes, mTORC1 (characterized by the presence of RAPTOR) and mTORC2 (characterized by the presence of RICTOR) (Benjamin et al., 2011). mTORC1 is the canonical target of rapamycin and is acutely inhibited by the drug, whereas mTORC2 is only disrupted after chronic rapamycin treatment in specific cell types and *in vivo* due to sequestration of the catalytic mTOR subunit (Lamming et al., 2012; Sarbassov et al., 2006; Schreiber, K. H. et al., 2015). mTORC1 is regulated by anabolic signals and amino acid availability to suppress autophagy and promote protein, lipid, and nucleic acid synthesis as well as nutrient transport (Kennedy et al., 2016). mTORC2 plays key roles in metabolism, cell survival and proliferation through multiple mechanisms including regulation of the insulin/IGF1 signaling cascade via phosphorylation of AKT at S473 (Kennedy & Lamming, 2016). Targeted disruption of mTORC1 or mTORC2 has

revealed that each complex has distinct and tissue-specific effects on signaling and metabolism (Kennedy & Lamming, 2016). Genetic or pharmacological targeting of the mTOR complexes has been shown to influence pathways that are important for whole body lipid homeostasis *in vivo*. These include changes in the expression of hepatic genes involved in lipogenesis and triglyceride (TG) secretion, suppression of lipoprotein lipase (LPL) activity, and over-activation of lipolysis via increased ATGL expression or PKA activity in adipocytes (Chakrabarti et al., 2013; Hagiwara et al., 2012; Houde et al., 2010; Kraemer et al., 1998; Soliman et al., 2010; Tory et al., 2008; Yecies et al., 2011; Yuan et al., 2012). However, the relative importance of these pathways and how they interact remain unclear. Indeed, an increase in circulating TG after mTOR inhibition is somewhat paradoxical, given that mTORC1 promotes lipogenesis, and genetic ablation of either mTOR complex in the liver results in unaltered or decreased plasma lipids, rather than an increase (Peterson, T. R. et al., 2011; Quinn et al., 2017; Yuan et al., 2012). In cultured adipocytes, mTORC1 inhibition decreases *de novo* lipogenesis and increases lipolysis, the latter proposed to be via either upregulation of ATGL expression or increased PKA-dependent phosphorylation of HSL (Chakrabarti et al., 2010; Chakrabarti et al., 2013; Soliman et al., 2010). After rapamycin treatment, the effects on lipolysis have been less clear, with increased circulating non-esterified fatty acids (NEFA) reported in some studies and decreased NEFA in others (Blanchard et al., 2012; Fang et al., 2018; Fang et al., 2013; Houde et al., 2010; Lopes et al., 2014). Decreased expression and activity of lipoprotein lipase have been more consistently observed after rapamycin treatment *in vivo* (Blanchard et al., 2012; Kraemer et al., 1998; Morrisett et al., 2003; Pereira et al., 2013; Tory et al., 2008). Genetic ablation of adipocyte mTORC1 with AP2-Cre resulted in lean mice that were protected from hypercholesterolemia

(Polak et al., 2008). However, this Cre can have off-target and mosaic effects (Lee, K. Y. et al., 2013). We and others have since generated a mouse model lacking Raptor specifically in adipocytes using adiponectin-Cre and reported increased (Tran, C. M. et al., 2016) or unchanged NEFA (Lee, P. L. et al., 2016). Lee et al. further described progressive lipodystrophy with increased de novo lipogenesis in adipocytes, and upward trends but no significant changes in cholesterol and TG (Lee, P. L. et al., 2016). In sum, the available data do not identify a clear or consistent role for adipocyte mTORC1 signaling in systemic lipid homeostasis, and prior studies have been somewhat confounded by concurrent lipodystrophy. Here we studied mice with adipose-specific *Raptor* ablation (*Raptor*<sup>akO</sup>) prior to the onset of lipodystrophy. We show that these animals display profound hypertriglyceridemia specifically in the fed state. Although lipoprotein lipase expression is decreased, the effect size is small, and this change alone does not explain the lipidomic profile in adipose tissue, which favors accumulation of di- and monoacylglycerols. While, *Raptor*<sup>akO</sup> mice have lower NEFA than controls during fasting, they have higher NEFA in the fed state, suggesting a failure to appropriately suppress lipolysis and potentially explaining why *in vivo* studies on rapamycin have reached opposite conclusions with respect to its effects on circulating NEFA. Strikingly, genetic ablation of ATGL to limit adipocyte lipolysis largely restored triglyceride homeostasis without correcting the deficiency in lipoprotein lipase expression. These results suggest that unrestrained lipolysis is the primary defect in hyperlipidemia induced by adipocyte mTORC1 inhibition *in vivo*.

## 2.3 Results

### *2.3.1 Adipocyte-specific loss of mTORC1 activity elevates plasma lipids prior to lipodystrophy*

To investigate the role of adipose mTORC1 on plasma lipid metabolism, we generated mice with adipose-specific deletion of Raptor (Raptor<sup>akO</sup>), which encodes an essential subunit of mTORC1. Deletion of Raptor in inguinal white adipose tissue (IWAT), epididymal white adipose tissue (EWAT) and brown adipose tissue (BAT), along with the expected reduction in phosphorylation of mTORC1 targets, ribosomal protein S6 and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) was confirmed by western blot (Figure 2.1A,B, Supplementary Fig. 2.1A-C). We also observed an increase in phosphorylation of AKT as expected due to the loss of negative feedback on the insulin signaling cascade (Huang et al., 2009). Deletion of Raptor did not change body weight in male (Figure 2.1C) or female mice (Supplementary Figure 2.1D), despite the onset of lipodystrophy by about 3 months of age, as reported previously (Lee, P. L. et al., 2016). We noted elevated expression of macrophage markers in EWAT (Supplementary Figure 2.1F) and relative adipose mass decreased with age in both genders, while liver mass increased (Figure 2.1D, Supplementary Figure 2.1E). In contrast to the mild or protective lipid phenotypes observed in previous studies, we noted consistent hypertriglyceridemia in Raptor<sup>akO</sup> mice (Supplementary Figure 2.1G,H). To avoid the confounding effects of decreased adipose tissue mass, we studied mice at ~10 weeks of age, a time point at which the sizes of adipose depots were similar between genotypes (Figure 2.1E). At this age, we also saw no evidence of hepatic steatosis (Supplementary Figure 2.1I). We found that random fed plasma cholesterol

and triglycerides were increased in Raptor<sup>akO</sup> compared to controls, and that a trend toward increased non-esterified fatty acids (NEFA) was also apparent (Figure 2.1F). We conclude that Raptor<sup>akO</sup> mice develop hyperlipidemia prior to lipodystrophy.

### *2.3.2 Plasma lipid levels are sensitive to adipocyte mTORC1 status in the fed state*

Hyperlipidemia has not been reported in previous studies of mice lacking adipocyte mTORC1 signaling, and conflicting reports exist as to the effect of pharmacologic mTORC1 inhibition on NEFA. To determine whether changes in feeding state might explain these apparent contradictions, we subjected mice to an overnight fast and subsequent four-hour period of refeeding. We found that the effect of adipocyte mTORC1 ablation on circulating TG is entirely feeding state-dependent. In the fasted state, plasma TG were similar between Raptor<sup>akO</sup> mice and controls, whereas in the refed state Raptor<sup>akO</sup> mice displayed profound elevations in circulating TG (Figure 2.2A). Moreover, Raptor<sup>akO</sup> mice displayed significantly lower NEFA in the fasted state, when lipolysis is normally activated, but significantly higher NEFA in the fed state, when lipolysis would normally be repressed (Figure 2.2B). Compared to controls, Raptor<sup>akO</sup> mice gained a similar amount of weight during refeeding and maintained similar blood glucose levels, suggesting that the altered lipid parameters were not due to changes in food intake (Supplementary Figure 2.2). We hypothesized that higher NEFA in the fed state might lead to an increase in re-esterification to TG in the liver and secretion as VLDL particles, which could explain the elevation of plasma TG. However, the rate of hepatic TG secretion (assessed by blocking lipase activity with nonionic surfactant P-407 and monitoring the rate of increase of plasma TG) was unchanged in Raptor<sup>akO</sup> mice in either the fasted or the fed state, despite higher levels of plasma TG before P-407

injection in the refed condition (Figure 2.2C). Next, we administered an oral fat tolerance test, which bypasses first-pass hepatic metabolism, to measure TG clearance.

Consistent with an extrahepatic cause for hypertriglyceridemia, Raptor<sup>akO</sup> mice displayed increased plasma TG compared to controls (Figure 2.2D). This finding indicates a defect in the clearance of plasma TG in Raptor<sup>akO</sup> mice.

### *2.3.3 Adipocyte-specific ablation of mTORC1 activity causes a modest deficiency in lipoprotein lipase expression*

To determine the cause of delayed clearance in the Raptor<sup>akO</sup> mice we examined lipoprotein lipase (LPL) gene expression and activity. LPL plays a central role in the clearance of lipid from plasma by hydrolyzing TG to NEFAs and monoacylglycerols (MG). LPL protein translocates from the cell of origin to the luminal surface of endothelial cells, where it is bound by proteoglycans and can act on circulating lipoprotein particles. LPL is active in adipose tissue after feeding, and can be released into the circulation by administration of heparin. We found that *Lpl* gene expression is decreased in IWAT and EWAT from Raptor<sup>akO</sup> mice (Figure 2.3A). Consistent with a defect in adipocyte *Lpl* expression in Raptor<sup>akO</sup> mice, we observed a decrease in heparin-releasable LPL activity in the fed state, but not during fasting (Figure 2.3B). Direct assay of LPL activity extracted from tissue lysates revealed decreases in the IWAT and BAT of refed Raptor<sup>akO</sup> mice (Figure 2.3C). In contrast, we were not able to measure a defect in EWAT, despite the reduction in *Lpl* mRNA level. Although the reason for this discrepancy is not clear, we speculate that reduced shedding of LPL into the bloodstream may help maintain tissue levels in the Raptor<sup>akO</sup> mice. Overall, decreases in LPL activity were significant, but generally mild in the Raptor<sup>akO</sup> mice. Thus, we sought



to test whether other factors might also contribute to the hyperlipidemia and lipodystrophy in Raptor<sup>akO</sup> animals.

#### *2.3.4 Lipidomic profiling reveals accumulation of lipolytic intermediates in adipose-specific mTORC1 KO mice*

To gain further insight into the alterations in adipocyte lipid metabolism that occur in the absence of mTORC1 signaling, we assayed lipidomic profiles in adipose from Raptor<sup>akO</sup> mice and wild type littermates in the refed state at 10 weeks of age. Simple inhibition of LPL would be expected to decrease the abundance of NEFA, monoacylglycerols (MG) and diacylglycerols (DG) in adipose tissue, since fewer of these metabolites would be released from circulating TG and available for re-esterification. In contrast, the lipidomic profiles revealed an increase in the abundance of MG and DG species, and a decrease in total TG content in Raptor<sup>akO</sup> adipose (Figure 2.4 A-E). Relative abundances for each lipid species in Raptor<sup>fl/fl</sup> vs. Raptor<sup>akO</sup> mice are listed in Supplementary Table 2.1. The increases in MG and DG suggest that either re-esterification is impaired or lipolysis is activated. These possibilities can be distinguished because lipolysis results in the release of free glycerol, whereas LPL-dependent hydrolysis of TG leaves the glycerol moieties trapped as MG. We found that Raptor<sup>akO</sup> mice in the refed state exhibited elevated glycerol concentrations, pointing to increased lipolysis as a driver of elevated NEFA in this model (Figure 2.4F).

#### *2.3.5 Genetic inhibition of lipolysis ameliorates hyperlipidemia in adipose-specific mTORC1 KO mice*

To limit lipolysis in the context of Raptor deletion, we concurrently deleted adipocyte triglyceride lipase (ATGL), which encodes the rate-limiting enzyme for hydrolysis of

adipocyte TG during lipolysis (Figure 2.5A, B). ATGL protein levels in the liver were not altered upon adipose specific ATGL deletion (Supplementary Figure 2.3A). Although body weight was unchanged, IWAT, EWAT and BAT weights were increased in both ATGL<sup>akO</sup> and ATGL-Raptor<sup>akO</sup> mice (Supplementary Figure 2.3B,C). ATGL loss restored refeed plasma NEFA to normal levels in Raptor<sup>akO</sup> mice *in vivo* (Figure 5C) and blocked the increase in basal lipolysis observed explants derived from Raptor<sup>akO</sup> EWAT (Figure 2.5D). ATGL loss also prevented the induction of lipolysis by CL316,243 in both genotypes. In addition to the expected effects on lipolysis, ATGL deletion prevented the increase in refeed plasma TG levels in Raptor<sup>akO</sup> mice (Figure 2.5E). Plasma cholesterol was also restored to normal levels (Supplementary Figure 2.3D). To exclude potential consequences of chronic deletion in the genetic models, we tested the effects of acute inhibition of mTORC1 and ATGL on *ex vivo* lipolysis. Inhibition of mTORC1 with rapamycin increased basal lipolysis and the addition of the ATGL inhibitor atglstatin was able to prevent the increase (Figure 2.5F). Notably, ATGL-Raptor<sup>akO</sup> mice did not have significantly altered *Angptl4* mRNA as compared to controls, and had the same reductions in *Plin1* and *Lpl* mRNA that were observed in Raptor<sup>akO</sup> (Supplementary Figure 2.3E,F). This suggests the possibility that unrestrained lipolysis, rather than a defect in LPL expression, is the primary cause of impaired lipid clearance in Raptor<sup>akO</sup> mice, consistent with the suggestion that LPL might be subject to feedback inhibition by NEFA and MG (Amri et al., 1996; Bengtsson et al., 1980; Saxena et al., 1989). However, mice lacking adipocyte ATGL have decreased hepatic TG stores, and lower plasma TG levels during fasting. Therefore, it could also be speculated that this decrease in fasting plasma TG might buffer the increase upon refeeding. To more directly test the ability of ATGL-Raptor<sup>akO</sup> mice to clear a fixed load of TG, we performed an oral fat tolerance

test. Concurrent loss of ATGL restored the ability of Raptor<sup>akO</sup> mice to clear exogenous TGs, limiting the increase in plasma TG to that seen in floxed littermates (Figure 2.5G). Although starting TG levels were lower with loss of ATGL, the rate of clearance and area under the curve were comparable to those of wildtype mice (Figure 2.5H). Thus, unrestrained lipolysis impairs clearance circulation of TG-rich lipoproteins in Raptor<sup>akO</sup> mice.

#### *2.3.6 Stimulating lipolysis is sufficient to delay clearance of plasma TG in WT mice*

Our results to this point demonstrated that lipolysis drives hyperlipidemia in the context of Raptor<sup>akO</sup> mice, which have multiple alterations in lipid-handling pathways. To determine whether increased lipolysis per se is sufficient to impair the clearance of TG in wild type mice, we performed an oral fat tolerance test with or without stimulation of lipolysis with the  $\beta$ 3-adrenergic agonist CL316,243. CL316,243 injection substantially increased plasma NEFA, as expected (Figure 2.6A). In mice that received the CL316,243 injections, clearance of the exogenous TG was also dramatically slowed, resulting in hyperlipidemia (Figure 2.6B). In contrast, CL316,243 did not increase plasma NEFA or plasma TG in mice lacking ATGL (Supplementary Figure 2.4). We next measured hepatic TG secretion to test whether CL316,243 could be slowing the apparent TG clearance rate by enhancing hepatic re-esterification of NEFA and packaging into TG-rich lipoproteins. Contrary to this hypothesis, we found that when peripheral TG uptake was blocked by treatment with P-407, CL316,243 actually decreased the rate of hepatic TG secretion and lowered plasma TG (Figure 2.6 C,D). These data indicate that CL316,243 increases plasma TG by delaying LPL-dependent clearance, rather than by increasing hepatic TG secretion.

### 2.3.7. *Inhibiting lipolysis restores plasma TG levels increased by rapamycin treatment*

We next investigated whether inhibiting adipocyte lipolysis *in vivo* is sufficient to reverse rapamycin-induced hyperlipidemia. Wildtype mice were treated with rapamycin (2 mg/kg daily by intraperitoneal injection) for 2 weeks to induce hypertriglyceridemia. To inhibit lipolysis selectively in adipose tissue, we used a GPR81 agonist (3CI-5OH-BA). GPR81 is predominantly expressed in adipose tissue, and physiological activation of this receptor by lactate leads to suppression of lipolysis (Ge et al., 2008; Sakurai et al., 2014). This approach avoids inhibiting hepatic ATGL, which complicates the interpretation of experiments targeting that enzyme pharmacologically. As expected, rapamycin-treated mice failed to fully suppress plasma NEFA levels upon refeeding, and this was corrected by treatment with the GPR81 agonist (Figure 2.7A). In parallel, the GPR81 agonist restored refeed plasma TG to control levels (Figure 2.7B). These results indicate that restoring adipocyte lipolysis to normal levels can mitigate rapamycin-induced hypertriglyceridemia.

## 2.4 Discussion

Although mTORC1 is known to influence lipid metabolism through multiple pathways in multiple cell types, the precise etiology of hyperlipidemia induced by systemic mTORC1 inhibition *in vivo* remains uncertain. Indeed, genetic loss of mTORC1 in hepatocytes causes the opposite phenotype, lowering circulating cholesterol and TG (Peterson, T. R. et al., 2011; Quinn et al., 2017). Here we show that loss of mTORC1 in adipocytes is sufficient to cause hypertriglyceridemia despite having only modest effects on the expression and *ex vivo* activity of lipoprotein lipase. Mechanistically, we show that although mTORC1 signaling affects many aspects of adipocyte biology,

hypertriglyceridemia is driven primarily by unrestrained lipolysis in the fed state, which likely interferes with the *in situ* activity of lipoprotein lipase.

Studies in cultured adipocytes have demonstrated that inhibition of mTORC1 signaling with rapamycin leads to increased lipolysis in response to  $\beta$ -adrenergic stimulation via enhanced HSL phosphorylation (Soliman et al., 2010), and more generally via activation of Egr1-dependent ATGL transcription (Chakrabarti et al., 2010; Chakrabarti et al., 2013). Here, we show that genetic deletion of ATGL in adipocytes is sufficient to normalize re-fed NEFA and TG levels in Raptor<sup>aKO</sup> mice, supporting a model in which lipolysis drives hypertriglyceridemia. However, we did not observe a significant increase in ATGL protein expression in adipose depots of Raptor<sup>aKO</sup> mice, consistent with several other studies (Lee, P. L. et al., 2016; Lopes et al., 2014; Pereira et al., 2013; Soliman et al., 2010). This suggests that ATGL expression *per se* cannot account for the increase in lipolysis. A more general mechanism that can account for the widespread effects of Raptor deletion on lipid metabolism and might contribute to increased lipolysis is the suppression of PPAR $\gamma$  and C/EBP $\alpha$  transcriptional activity in the absence of mTORC1 (Blanchard et al., 2012; Kim, J. E. & Chen, 2004; Lee, P. L. et al., 2016). The phenotypes of adipose-specific C/EBP $\alpha$  KO mice are complicated by severe lipodystrophy early in life, but we note that these animals exhibit impaired TG clearance (Chatterjee et al., 2011). Germline ablation of the C/EBP $\alpha$  target *Plin1* (encoding perilipin 1) mimics the lipolytic phenotype of Raptor<sup>aKO</sup> mice; these animals have increased basal lipolysis and decreased stimulated lipolysis (Tansey et al., 2001). Moreover, decreased expression of *Plin1* is observed in mice treated with rapamycin (Pereira et al., 2013) and in Raptor<sup>aKO</sup> mice (Supplementary Figure 2.3F). Perilipin 1

coats the outside of lipid droplets to prevent hydrolysis by ATGL. Thus, a decrease in perilipin 1 expression is one mechanism that could account for an increase in ATGL-dependent lipolysis in the absence of a change in ATGL expression.

A number of prior studies have implicated decreased adipose LPL in the hypertriglyceridemia associated with rapamycin treatment (Blanchard et al., 2012; Kraemer et al., 1998; Morrisett et al., 2003; Pereira et al., 2013; Tory et al., 2008). Consistent with these reports, we observed a decrease in LPL gene expression, and showed that TG clearance, but not hepatic production of TG-rich lipoproteins, is altered in Raptor<sup>aKO</sup> mice. However, we were able to detect only small decreases in the LPL activity that could be released by heparin *in vivo* or extracted from adipose tissue. Moreover, deletion of ATGL restored TG clearance without correcting *Lpl* gene expression. LPL activity has been shown to be inhibited by ANGPTL4, *in vitro* and *in vivo*, which could account for differences in activity vs. expression (Koster et al., 2005; Sukonina et al., 2006). However, we were unable to detect consistent changes in *Angptl4* expression in Raptor<sup>aKO</sup> mice, regardless of ATGL status (Supplementary Figure 2.3F). Another possibility to explain the disconnect between LPL expression and activity is inhibition due to local accumulation of NEFA and MG. It has long been appreciated that NEFA can suppress LPL through multiple mechanisms, including product inhibition, displacement of the active enzyme from the endothelium, and preventing its interaction with activating factors such as ApoC-II (Amri et al., 1996; Gordts et al., 2016; Peterson, J. et al., 1990; Saxena et al., 1989). As a “proof of principle” experiment for this mechanism, we stimulated adipose tissue lipolysis with a  $\beta$ -adrenergic agonist and showed that this was sufficient to delay TG clearance in an oral fat tolerance test.

Similarly, a GPR81 agonist that suppresses adipocyte lipolysis was sufficient to acutely normalize plasma TG in rapamycin-treated mice. Notably, acute dosing with niacin has been reported to suppress postprandial hypertriglyceridemia in humans, and it was speculated that this might be related to a reduction in NEFA release from adipose (Montserrat-de la Paz et al., 2018; Usman et al., 2012). Thus, we propose that inappropriate lipolysis in the fed state directly impairs LPL activity in Raptor<sup>akO</sup> mice, and that such inhibition may be a general feature of hyperlipidemia.

An interesting aspect of the elevated NEFA and TG phenotypes of Raptor<sup>akO</sup> mice is their complete dependence on feeding status. This may provide an explanation for conflicting reports on the effect of rapamycin on NEFA, which have been significantly increased in some studies and decreased in others (Blanchard et al., 2012; Fang et al., 2018; Fang et al., 2013; Houde et al., 2010; Lopes et al., 2014). It also makes clear that impaired adipocyte mTORC1 signaling is not the only factor contributing to rapamycin-induced hyperlipidemia, since circulating TG levels in rapamycin-treated animals are not completely resolved by fasting. In part, this may reflect the ability of rapamycin to reduce LPL expression in tissues such as skeletal and cardiac muscle that play a larger role in TG clearance during fasting. We also note that chronic rapamycin treatment can impair mTORC2 activity; as mice lacking mTORC2 in adipose tissue have a defect in insulin-dependent suppression of lipolysis, both mTOR complexes may act together to fully restrain adipocyte lipolysis (Kumar et al., 2010). Consistent with a role for mTORC2 in rapamycin-induced hyperlipidemia, it was recently shown that a rapamycin derivative that avoids mTORC2 inhibition (DL001) also fails to induce hyperlipidemia in fasted mice (Schreiber, K. H. et al., 2019). The effect of DL001 on TG in fed mice has not been

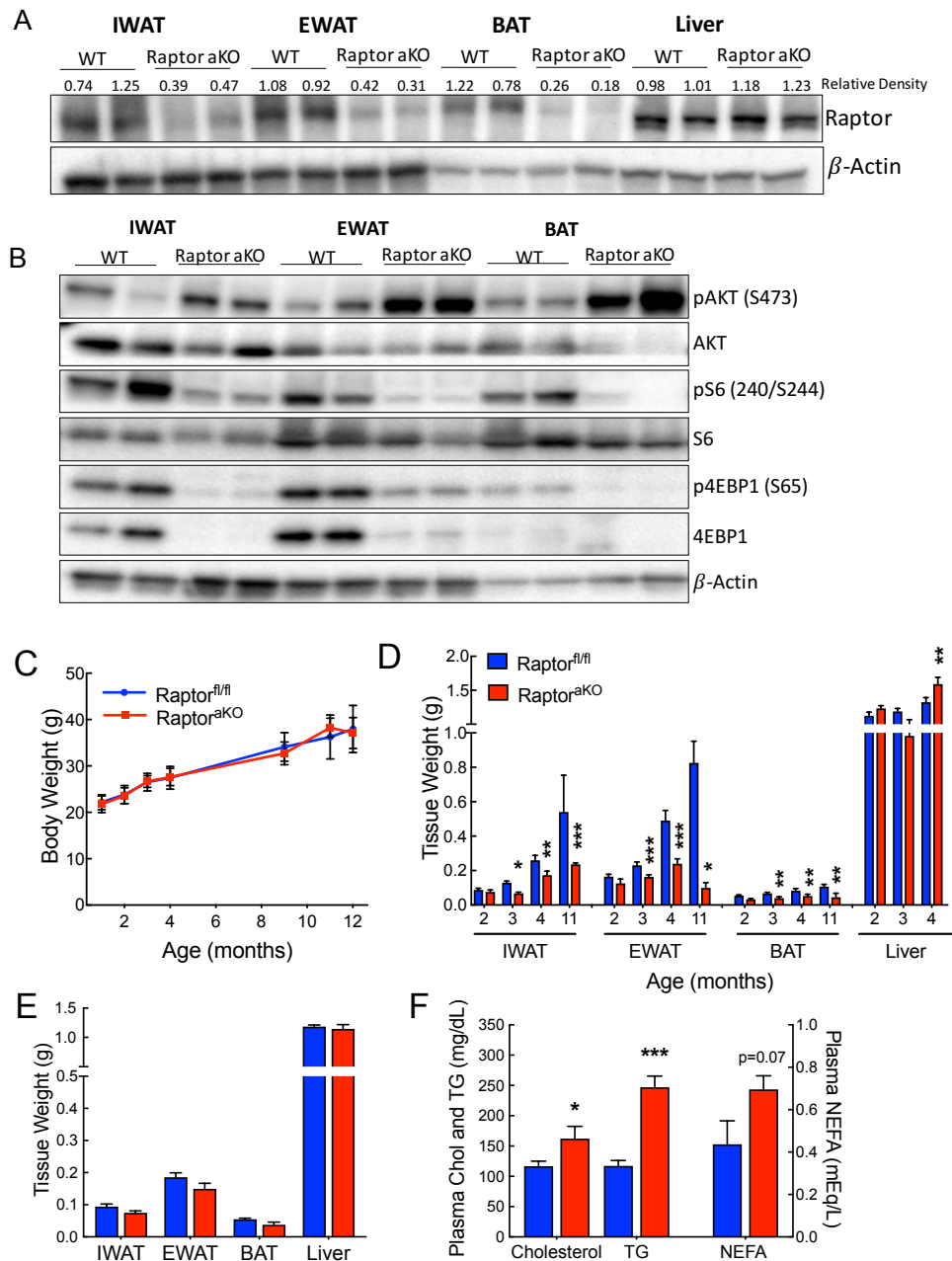
reported. Finally, although our data suggest that lipolysis can affect plasma cholesterol levels, it is unclear whether this is directly related to LPL activity, and further study will be required to fully elucidate the mechanism and the degree to which loss of adipocyte mTORC1 recapitulates the hypercholesterolemic effects of rapamycin.

We demonstrate that mTORC1 signaling in adipose tissue is critical for the maintenance of plasma lipid homeostasis in the fed state. Lack of mTORC1 in adipocytes increases both lipolysis and circulating TG in fed mice. Lipolysis is causally related to hypertriglyceridemia, since genetic ablation of ATGL resolves both circulating NEFA and TG clearance rates. Moreover, direct stimulation of lipolysis in wild type mice is sufficient to impair TG clearance. Thus, mTORC1-dependent suppression of adipocyte lipolysis is an essential switch to maintain plasma lipid concentrations during feeding.



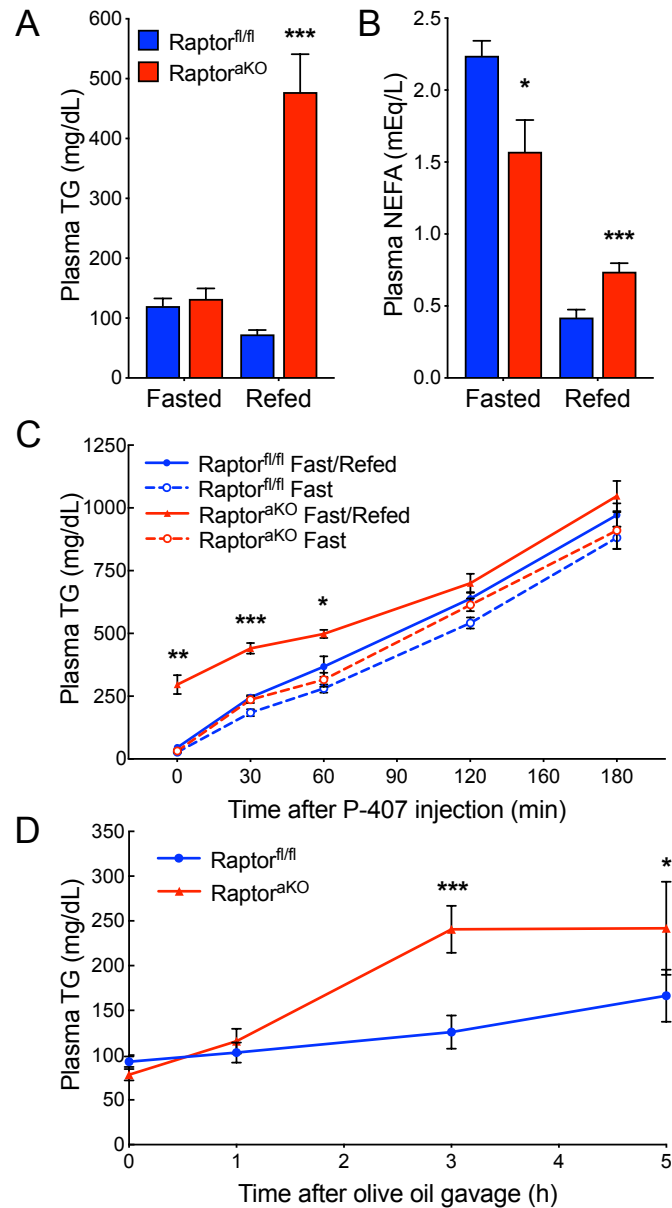
**Figure 2-1. Adipocyte-specific ablation of mTORC1 elevates plasma lipids prior to lipodystrophy.**

(A) Western blot for Raptor in IWAT, EWAT, BAT and liver. Numbers indicate relative density normalized to  $\beta$ -actin. (B) Western blot of downstream mTORC1 substrates in IWAT, EWAT, and BAT of Raptor<sup>akO</sup> and Raptor<sup>fl/fl</sup> littermates. (C) Body and (D) tissue weights with age of male Raptor<sup>akO</sup> mice compared to controls (male, n=3-11). (E) Tissue weights and (F) random fed plasma lipid parameters of 10-week-old Raptor<sup>akO</sup> mice (n=6-9). Data presented as mean  $\pm$  s.e.m. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



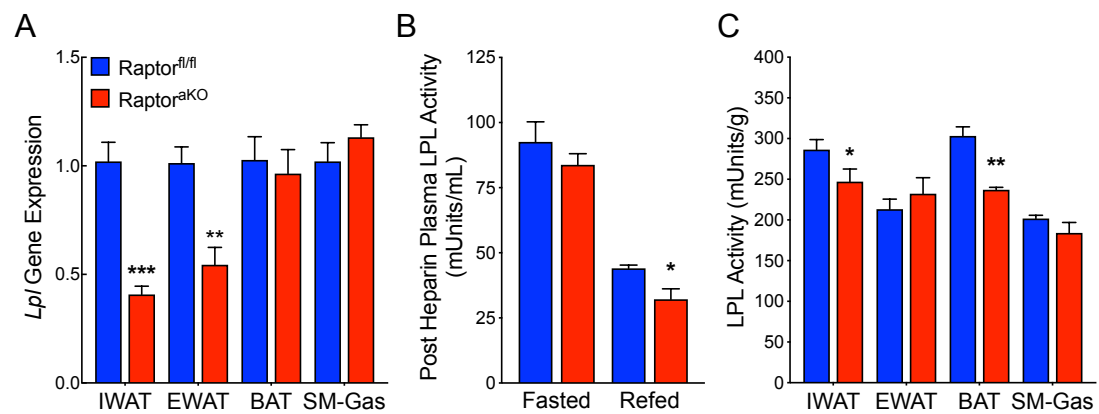
**Figure 2-2. Adipocyte-specific ablation of mTORC1 elevates plasma lipids upon refeeding.**

10-week-old Raptor<sup>fl/fl</sup> and Raptor<sup>akO</sup> mice were fasted overnight and refed. (A) Plasma TG (n=8-10). (B) Plasma NEFA (n=8-10). (C) Mice were fasted (n=5) or fasted and refed (n=3-4) as described in methods and plasma TG was measured at indicated time points after injection with P-407 to measure hepatic TG secretion. (D) Mice were fasted and plasma TG was measured at indicated time points during an oral fat tolerance test to measure clearance of circulation TG (n=6-8). Data presented as mean  $\pm$  s.e.m. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



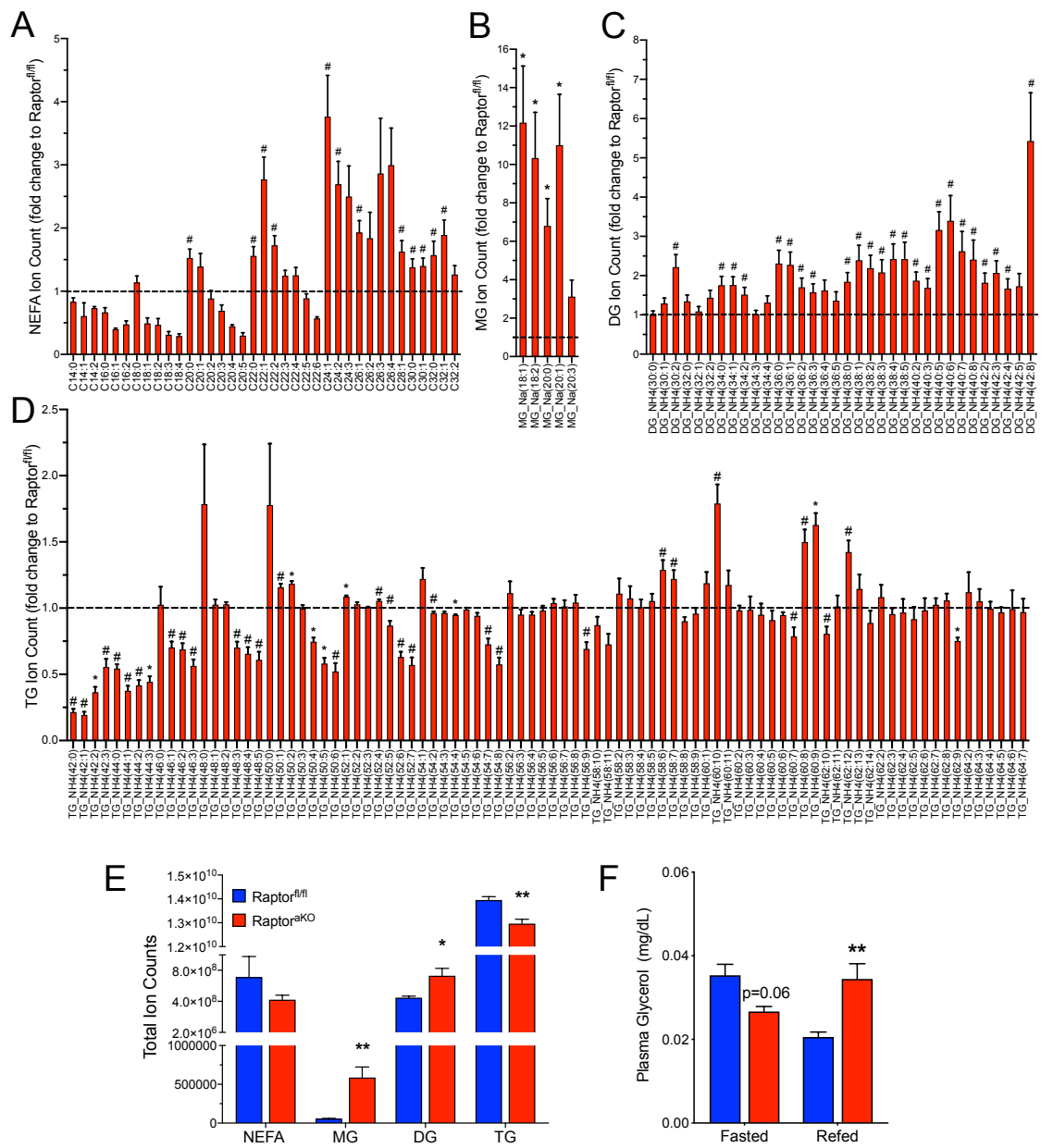
**Figure 2-3. Adipocyte-specific ablation of mTORC1 causes a modest deficiency in lipoprotein lipase.**

(A) *Lpl* gene expression normalized to TBP for IWAT, EWAT, BAT and skeletal muscle from refed 10-week-old Raptor<sup>fl/fl</sup> and Raptor<sup>akO</sup> mice (n=4-6). (B) Post-heparin plasma LPL activity in the fasted and refed state (n=6). (C) Tissue LPL activity for IWAT, EWAT, BAT and skeletal muscle (n=4). Data presented as mean  $\pm$  s.e.m. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



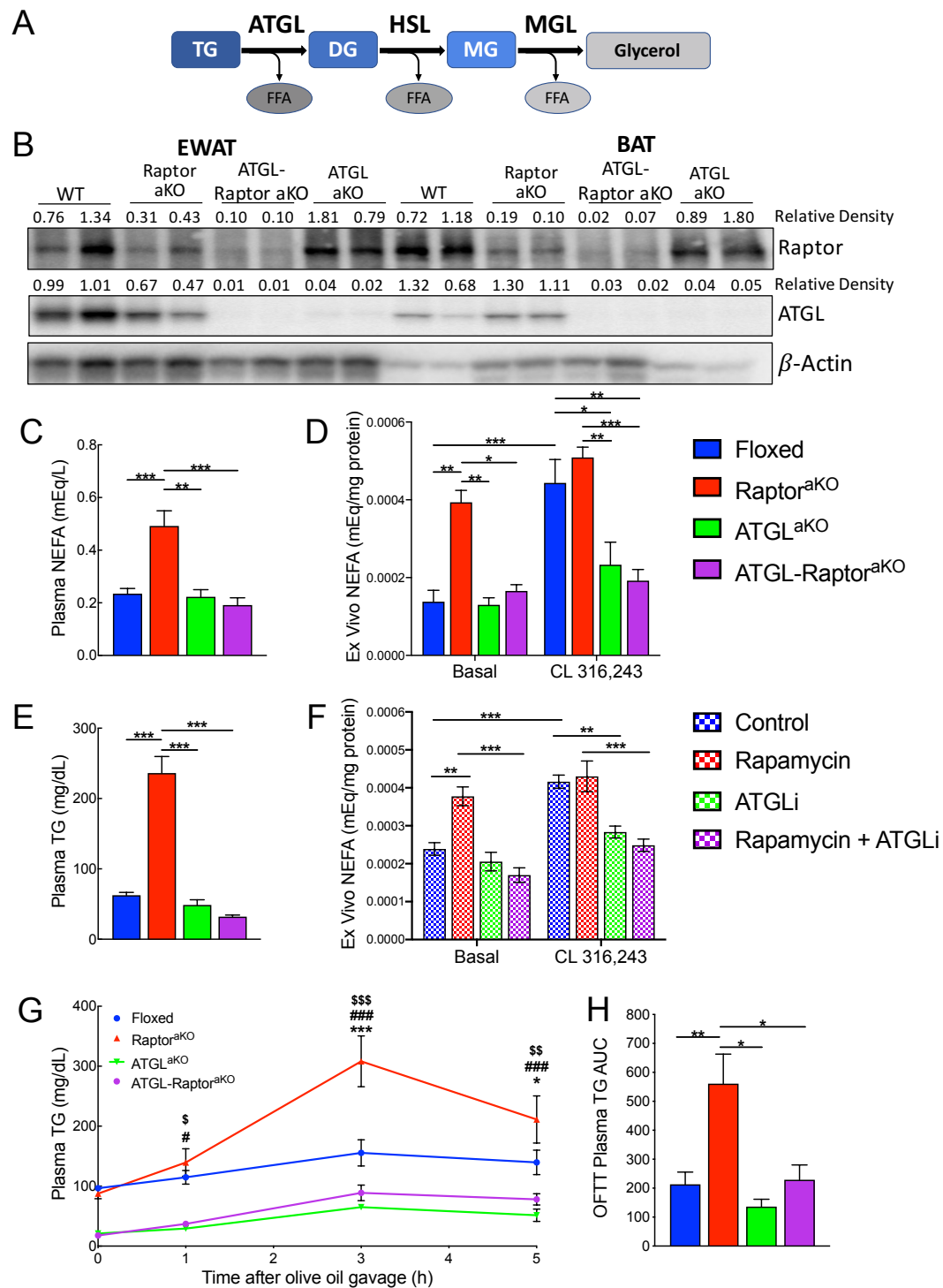
**Figure 2-4. Adipocyte-specific ablation of mTORC1 alters intracellular lipids.**

10-week-old Raptor<sup>fl/fl</sup> and Raptor<sup>AKO</sup> mice were fasted overnight and refed for 4 h prior to collection of EWAT for lipidomics. Fold change in relative abundance compared to Raptor<sup>fl/fl</sup> for (A) NEFA, (B) monoacylglycerols (MG), (C) diacylglycerols (DG), and (D) triglycerides (TG) (n=5-6). A-D: #p < 0.05 for two-tailed unpaired t-test prior to multiple comparison and \*p < 0.05 corrected for multiple comparison using the Holm-Sidak method. (E) Total ion counts from (A-D). (F) Plasma glycerol (n=3-6). Data presented as mean  $\pm$  s.e.m. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

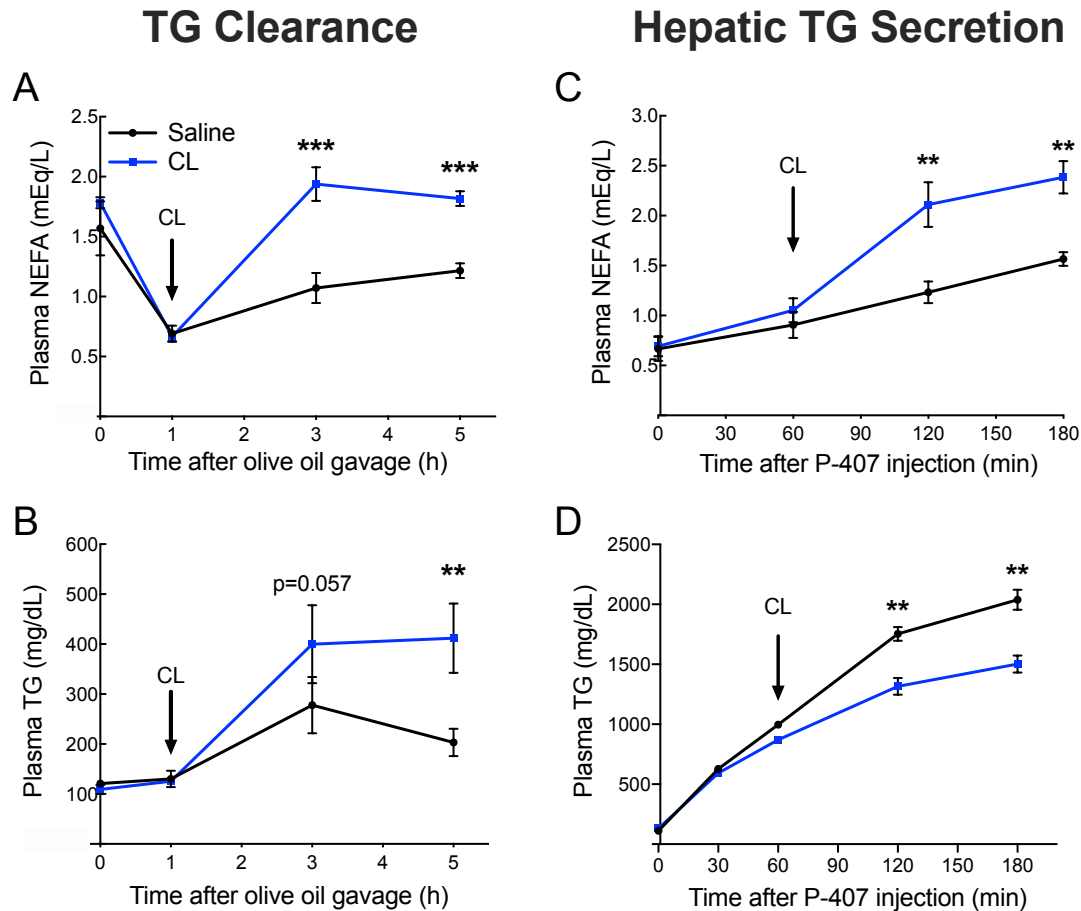


**Figure 2-5. Genetic inhibition of lipolysis reverses lipid phenotypes of adipose-specific mTORC1 KO.**

10-week-old mice were fasted overnight and refed. (A) Diagram of the lipolysis pathway. (B) Western blot and relative density (normalized to  $\beta$ -actin) of Raptor and ATGL protein from EWAT, BAT. (C) refed plasma NEFA (Floxed n=9, Raptor<sup>akO</sup> n=5, ATGL<sup>akO</sup> n=3, ATGL-Raptor<sup>akO</sup> n=9). (D) Refed plasma TG (Floxed n=9, Raptor<sup>akO</sup> n=5, ATGL<sup>akO</sup> n=3, ATGL-Raptor<sup>akO</sup> n=9). (E) NEFA from *ex vivo* EWAT lipolysis with or without stimulation by 1  $\mu$ M CL316,243 (Floxed n=10, Raptor<sup>akO</sup> n=4, ATGL<sup>akO</sup> n=5, ATGL-Raptor<sup>akO</sup> n=5). (F) *Ex vivo* lipolysis in EWAT from wildtype mice treated with 500 nm rapamycin and 100  $\mu$ m atglistatin (G) Plasma TG clearance during an oral fat tolerance test and (H) AUC (Floxed n=11, Raptor<sup>akO</sup> n=12, ATGL<sup>akO</sup> n=3, ATGL-Raptor<sup>akO</sup> n=11). Data presented as mean  $\pm$  s.e.m. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ . (G) Raptor<sup>akO</sup> compared to floxed, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ . Raptor<sup>akO</sup> compared to ATGL-Raptor<sup>akO</sup> # =  $p < 0.05$ , ## =  $p < 0.01$ , ### =  $p < 0.001$ . Raptor<sup>akO</sup> compared to ATGL<sup>akO</sup> \$ =  $p < 0.05$ , \$\$ =  $p < 0.01$ , \$\$\$ =  $p < 0.001$ .



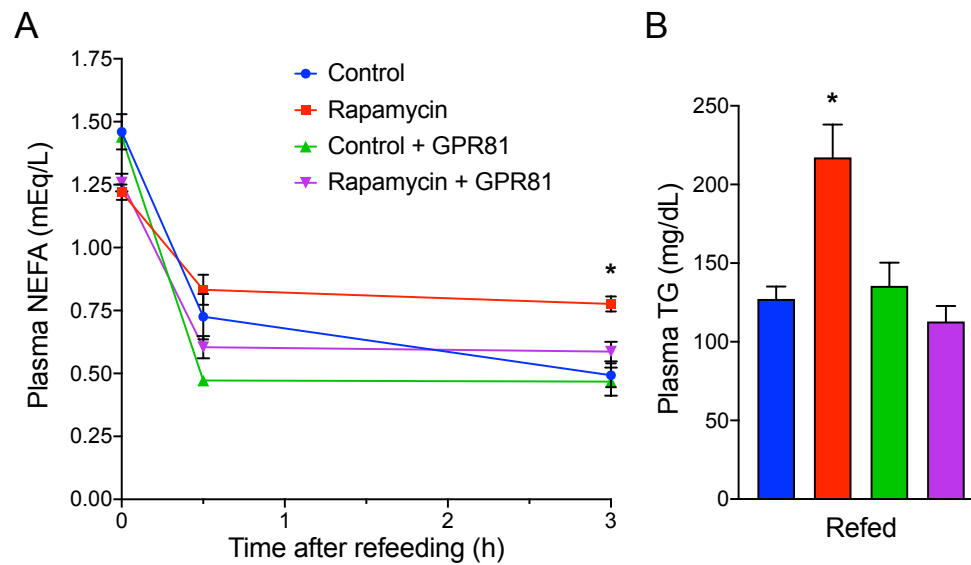
**Figure 2-6. Increased plasma NEFA from lipolysis delays clearance of plasma TG.**  
 (A, B) Male C57BL/6 mice were fasted overnight and gavaged with olive oil for an oral fat tolerance test. Mice were injected with either saline or CL316,243 at 1 hour (arrow). (A) Plasma NEFA and (B) plasma TG were measured at the indicated time points (n=5-6). (C, D) Mice were fasted overnight and refed then injected with P-407 to measure hepatic TG secretion. Either saline or CL316,243 was injected at 1 hour (arrow). (C) Plasma NEFA and (D) plasma TG was measured at indicated time points after P-407 injection. Data presented as mean  $\pm$  s.e.m. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.





**Figure 2-7. Inhibiting lipolysis restores plasma TG levels in rapamycin treated mice.**

Male C57BL/6 mice were treated with 2mg/kg rapamycin for 2 weeks to induce hyperlipidemia. Mice were fasted overnight then injected with 100 mg/kg GPR81 agonist at the time of refeeding. (A) Plasma NEFA and (B) plasma TG were measured at 0, 0.5 and 3 h after refeeding. Data presented as mean  $\pm$  s.e.m. Compared to rapamycin treatment, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

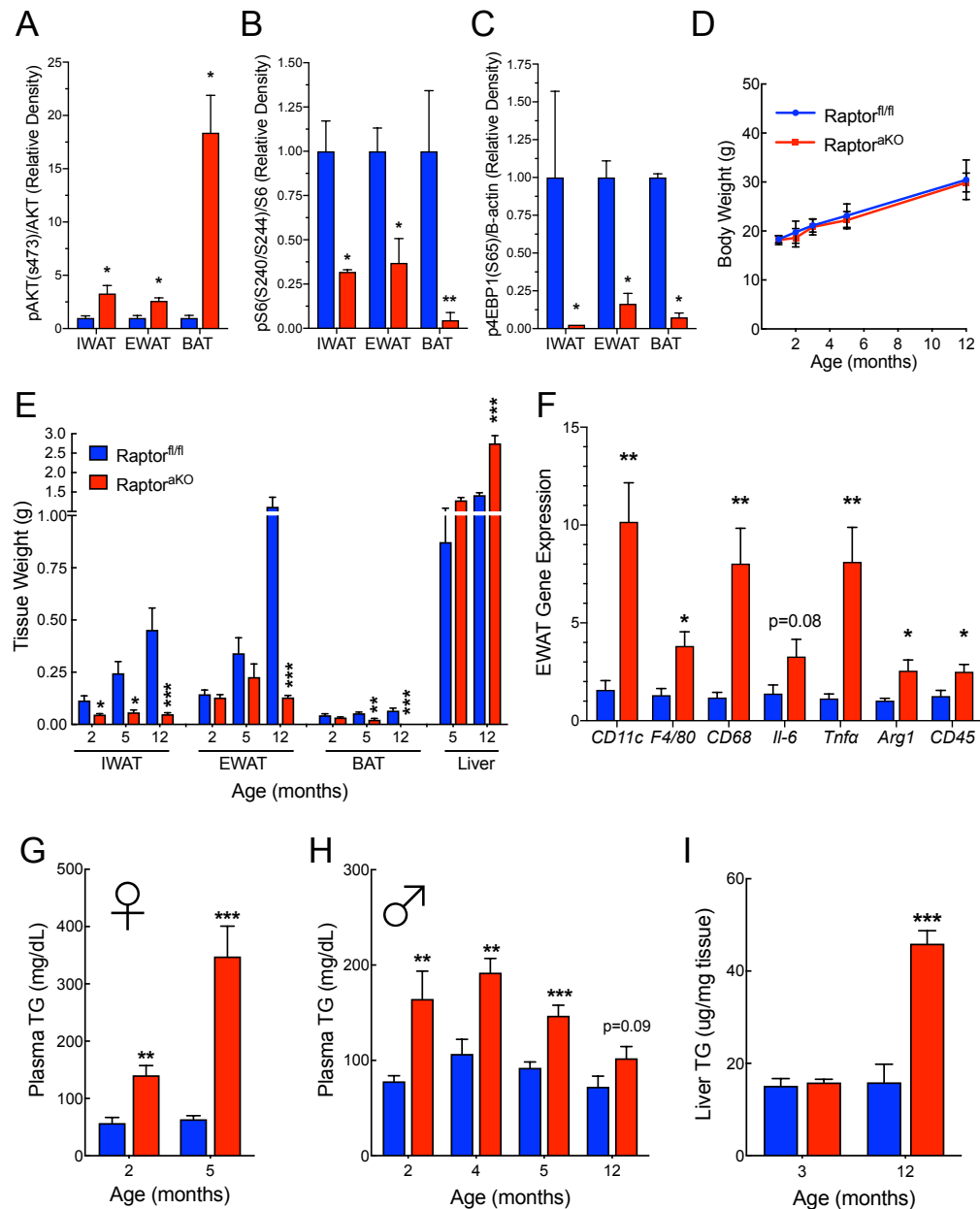


**Supplementary Table 2-1. EWAT lipidomics ion counts for Raptor<sup>fl/fl</sup> and Raptor<sup>akO</sup> mice.**

Data presented as mean  $\pm$  s.e.m. for each genotype (n=5-6). Two-tailed unpaired t-test. P-value prior to multiple comparison and adjusted p-value corrected for multiple comparison using the Holm-Sidak method.

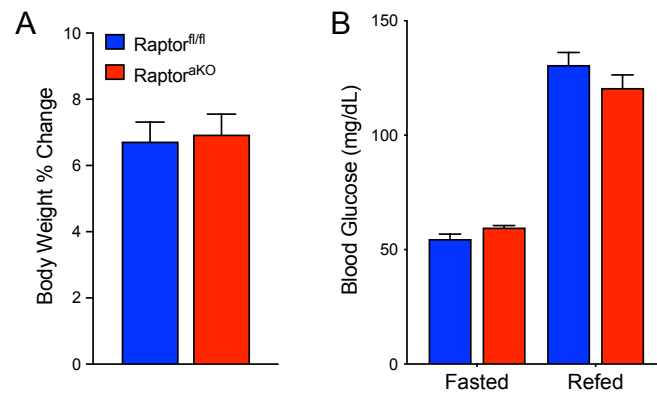
# **Supplementary Figure 2-1. Adipocyte-specific ablation of mTORC1 elevates plasma lipids prior to lipodystrophy.**

(A-C) Relative density of western blots from Figure 1B (n=2). (D) Body and (E) tissue weights with age of female Raptor<sup>fl/fl</sup> mice compared to controls (n=4-15). (F) Immune cell gene expression in EWAT from 10 week old males (n=5). Random fed plasma TG from females (G) and males (H) (females n=4-12, males n=6-10). (I) Liver TG from male Raptor<sup>fl/fl</sup> mice compared to controls at 3 and 12 months (Raptor<sup>fl/fl</sup> n=5-10, Raptor<sup>fl/fl</sup> n=6). Data presented as mean + s.e.m. Two-tailed unpaired t-test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



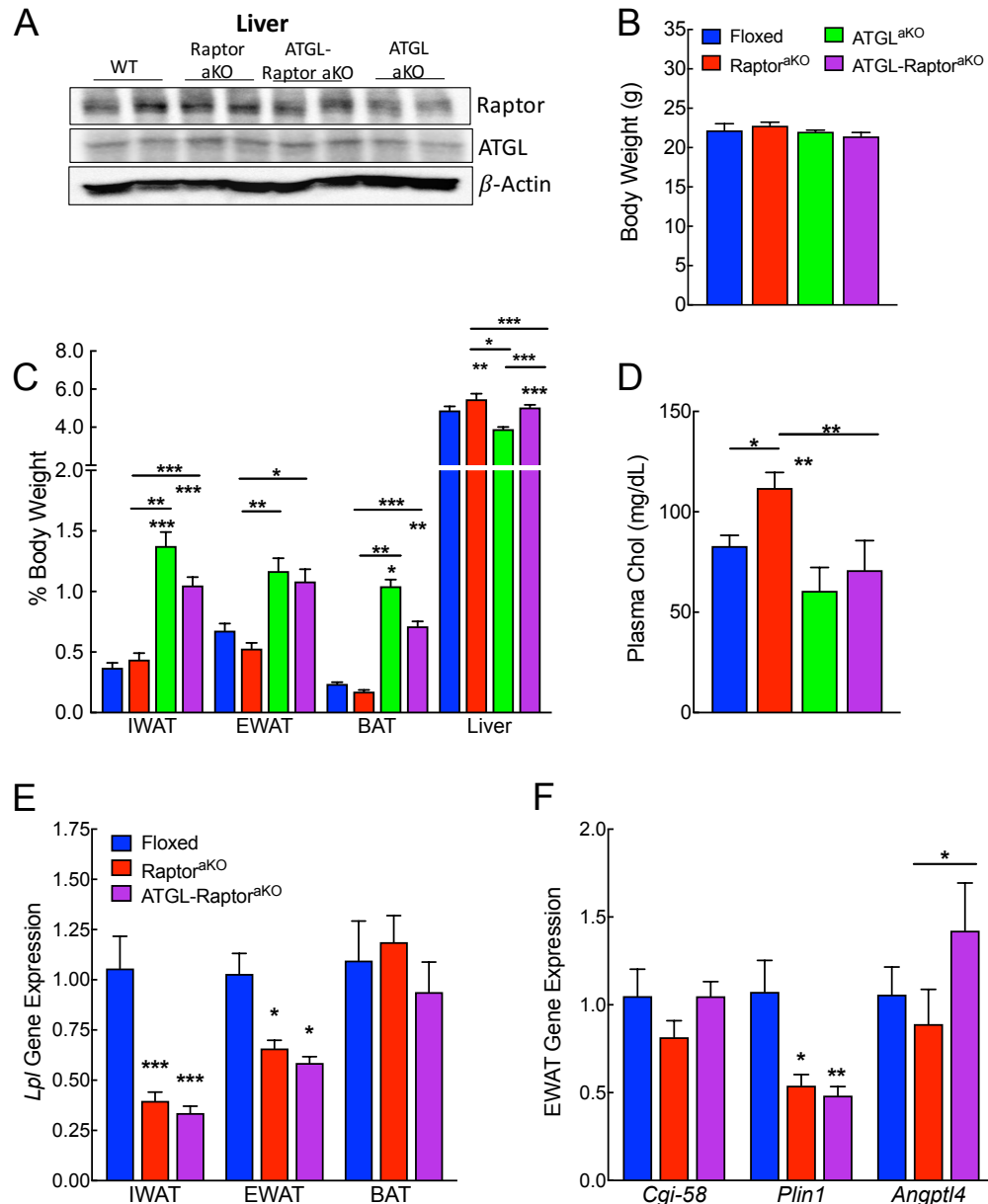
**Supplementary Figure 2-2. Adipocyte-specific ablation of mTORC1 elevates plasma lipids upon refeeding.**

10-week-old Raptor<sup>fl/fl</sup> and Raptor<sup>aKO</sup> mice were fasted overnight and refed. (A) Change in body weight with refeeding and (B) Blood glucose (n=8-10). Data presented as mean  $\pm$  s.e.m. Two-tailed unpaired t-test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



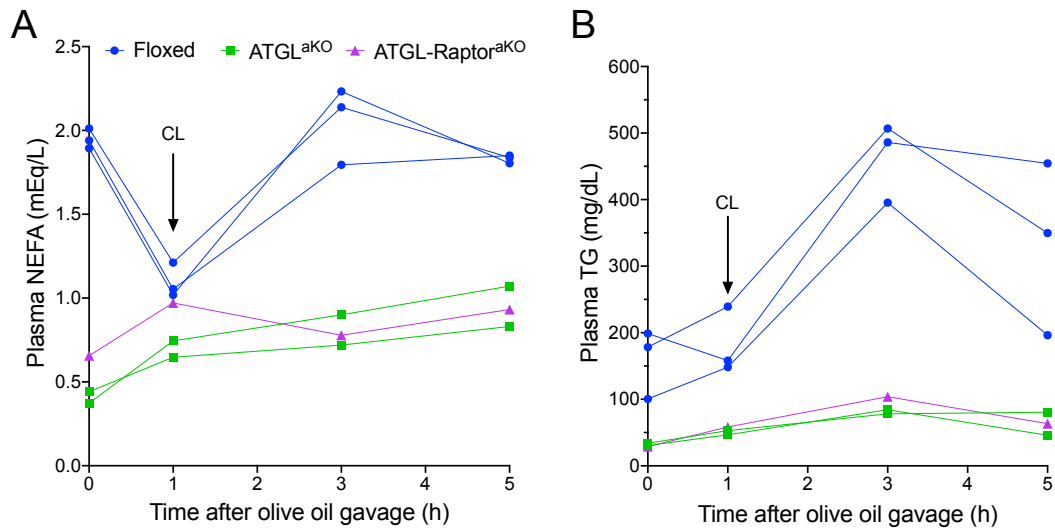
### Supplementary Figure 2-3. Genetic inhibition of lipolysis reverses lipid phenotypes of adipose-specific mTORC1 KO mice.

(A) Western blot of Raptor and ATGL protein in liver (n=2). 10-week-old mice were fasted overnight and refed. (B) Body weight and (C) IWAT, EWAT, BAT and liver weight as percent body weight (n=3-6). (D) Refed plasma cholesterol (n=3-9). (E) *Lpl* gene expression from IWAT, EWAT and Bat (n=6-8). (F) *Cgi-58*, *Plin1* and *Angptl4* gene expression from EWAT (n=5). Data presented as mean  $\pm$  s.e.m. Two-way ANOVA corrected for multiple comparisons using the Tukey method. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .



**Supplementary Figure 2-4. Delayed clearance of plasma TG after  $\beta$ -adrenergic stimulation requires adipocyte lipolysis.**

Mice were fasted overnight and gavaged with olive oil for an oral fat tolerance test. All mice were injected with CL316,243 at 1 hour (arrow). (A) Plasma NEFA and (B) plasma TG were measured at the indicated time points. Data presented as individual data points.



# **CHAPTER 3: Loss of mTOR signaling in adipose tissue does not solely account for the hyperlipidemia induced by rapamycin treatment**

### 3.1 Abstract

Rapamycin has been shown to expand median and maximal life span in mice, yet induces hyperlipidemia. Raptor and Rictor are essential component of mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) and 2 (mTORC2), respectively. Loss of mTORC1 signaling in adipose is sufficient to disrupt lipid homeostasis, resulting in hyperlipidemia caused by unrestrained lipolysis. However, studies to date examining the role of deletion of this complex, complex 2 or both in adipose tissue in combination with rapamycin have yet to be investigated. Here, we report the consequences of Raptor, Rictor or both deleted specifically in mature adipocytes driven by Adiponectin-Cre (Raptor<sup>AKO</sup>, Rictor<sup>AKO</sup>, Raptor-Rictor<sup>AKO</sup>). Concordant with the Raptor<sup>AKO</sup> mice, Rictor<sup>AKO</sup> mice display pronounced hyperlipidemia and both KO models have a further increase in plasma lipids with rapamycin treatment. Genetic inhibition of lipolysis in mice with loss of mTORC1 (ATGL-Raptor<sup>AKO</sup>) treated with rapamycin prevents the further increase in plasma lipids seen in Raptor<sup>AKO</sup> mice treated with rapamycin. We propose a general mechanism that in the fed state, rapamycin inhibition of adipose mTORC1 leads to decreased C/EBP $\alpha$  transcriptional activity. This decreased transcriptional activity results in decreased expression of perilipin and subsequent unrestrained lipolysis, leading to hyperlipidemia.



### 3.2 Introduction

Rapamycin has recently been shown to increase life span and delay age related changes in mice, raising the possibility that it could be more broadly useful in humans. However, there are metabolic side effects associated with rapamycin treatment that pose significant challenges to its therapeutic use for age-related effects in otherwise healthy individuals. The mechanism(s) by which rapamycin treatment causes hypercholesterolemia and hypertriglyceridemia are not known. Hyperlipidemia is a major risk factor for cardiovascular diseases due to the acceleration of plaque formation. The use of lipid-lowering drugs in combination with rapamycin does not reduce fasting plasma cholesterol levels below the recommended target level (Firpi et al., 2004; Lisik et al., 2007). Understanding how rapamycin treatment causes the adverse side effects of elevated plasma cholesterol and TGs will improve our basic understanding of lipid metabolism and allow the development of strategies to mitigate this process and to make use of the therapeutic properties of rapamycin in humans

The discovery of rapamycin's antiproliferative and immunosuppressive properties provoked a significant effort to identify the mode of action. This led to the identification of target of rapamycin (TOR) proteins in yeast in 1991 and identification of their mammalian counterpart, known as mechanistic target of rapamycin (mTOR) in 1994 (Chiu et al., 1994; Sabatini et al., 1994). mTOR is a Ser/Thr protein kinase that controls cell growth and metabolism. mTOR nucleates two structurally and functionally distinct complexes, mTORC1 (characterized by the presence of raptor) and mTORC2 (characterized by the presence of rictor). mTORC1 is the canonical target of rapamycin and is acutely inhibited by the drug, whereas mTORC2 is not affected by short-term

exposures (up to several hours). In 2012, the Baur and Sabatini labs demonstrated that chronic rapamycin treatment disrupts mTORC2 assembly in liver, white adipose tissue and skeletal muscle *in vivo*, suggesting a second molecular mechanism of rapamycin effects (Lamming et al., 2012; Schreiber, K. H. et al., 2015). Tissue specific disruption of mTORC1 or mTORC2 has revealed that each complex has different effects on whole body glucose and lipid homeostasis in different organs (Kennedy & Lamming, 2016).

Recent studies have revealed that mTOR signaling is critical in the regulation of adipose tissue function, including roles in adipogenesis, lipid metabolism and thermogenesis. We have previously reported the effects of adipose mTORC1 KO mice. Loss of adipose mTORC1 signaling increased plasma cholesterol, TG and NEFA and this effect is dependent on the fed state. In addition, we showed that the increase in plasma TG is caused by a failure to restrain adipose lipolysis, as mice with genetic inhibition of lipolysis restores plasma TG levels in Raptor<sup>akO</sup> mice. A more general mechanism that we propose to account for the widespread effects of Raptor deletion on lipid metabolism and might contribute to increased lipolysis is the suppression of C/EBP $\alpha$  transcriptional activity (Blanchard et al., 2012; Kim, J. E. & Chen, 2004; Lee, P. L. et al., 2016).

mTORC2, defined by the presence of the essential subunit Rictor (rapamycin-insensitive companion of mTOR) was more recently discovered and remains less well understood. Loss of mTORC2 function in the whole body, liver, or adipose tissue has been reported to disrupt glucose homeostasis, whereas its loss in skeletal muscle has comparatively mild effects. mTORC2 has emerged as a key controller of lipid metabolism in the liver and adipose tissue, but the mechanisms involved have yet to be

determined. Two studies that have examined the role of this complex in mature adipocytes both relied on *aP2/fabp4-Cre*, which can have off-target effects in macrophages and neurons that complicate the interpretation of phenotypes (Cybulski et al., 2009; Kumar et al., 2010). These groups observed profound hyperinsulinemia and insulin resistance, impaired suppression of lipolysis, and an increase in body and organ size that was attributed to an increase circulating IGF1. In contrast, deletion of Rictor in adipocyte precursors using *Myf5-Cre* was found to protect mice from obesity, enhance oxidative metabolism in brown adipocytes, and to promote the formation of smaller, multilocular cells resembling brown/beige adipocytes in white adipose depots (Hung et al., 2014). Two important caveats to this study were that effects on differentiation could not be separated from effects on mature adipocytes, and that loss of Rictor was mosaic in white adipose depots. Highlighting the importance of the timing of deletion, Albert et al. showed that in mature brown adipocytes (*Ap2-Cre* driven deletion) mTORC2-dependent glucose uptake was required to sustain thermogenesis (Albert et al., 2016).

Impressively, treatment with rapamycin can begin as late as 600 days in mice, which is comparable to 60 years of age in humans, suggesting that rapamycin may be able to treat age-related diseases and improve health in middle-aged humans. The effects of rapamycin on longevity are not restricted to cancer and immune effects. For instance, mTORC1 inhibition with rapamycin promotes the degradation of aggregate-prone proteins *in vitro*, reduces the severity of neurodegeneration *in vivo*, reducing the aggregation of misfolded proteins by slowing protein synthesis and/or promoting autophagy (Caccamo et al., 2010; Spilman et al., 2010). This suggests that mTORC1 signaling may play a role in the development of these pathologies. In fact, mTOR is

frequently dysregulated in human diseases, including cancer, obesity and type 2 diabetes and neurodegeneration. However, the use of rapamycin as a therapeutic for aging and age related diseases is limited by the hyperlipidemia and other metabolic side effects. This study will expand our knowledge on mTOR signaling in adipose tissue and the role of adipose tissue in metabolic dysregulation, and provide a mechanistic understanding of the hyperlipidemia seen with rapamycin treatment. Ultimately, this work has the potential to lead to improved therapeutic uses of rapamycin to improve quality of life for patients receiving the drug and to allow its use for the prevention or delay of age-related disorders in otherwise healthy people.

### **3.3 Results**

*3.3.1. Two weeks of rapamycin treatment in mice is sufficient to recapitulate the increase in plasma lipids that is seen in human.*

It has been shown that rapamycin increase plasma lipids in mice, as in humans. However, it remains unclear whether the low doses of rapamycin used for most of longevity studies induces the same metabolic changes associated with higher doses. Therefore, a lower dose was used in this study to tested whether our method can recapitulate the effects seen in humans. Using a dose of 2mg/kg rapamycin, which is similar to the doses used in aging studies, in a .25% Peg400/Tween80 vehicle in male mice, we showed that this 2 weeks of treatment does not affect body weight of tissues weights (Figure 3.1 A,B). In addition, rapamycin treatment was able to increase plasma lipids, including cholesterol (both HDL and non-HDL cholesterol), TG, phospholipids (PL) and NEFA (Figure 1C). In human studies, rapamycin increases different apolipoproteins. Although we did not test the different apolipoproteins in rapamycin treated me, we

performed FPLC on plasma to separate the different lipoproteins into fractions in order to measure the cholesterol and TG concentrations (Figure 1D). Rapamycin treatment increase lipids in CM/VLDL (5-10 fractions), IDL/LDL (15-30 fractions) and HDL (30-45 fractions). Since the plasma for FPLC was pooled from multiple mice, statistics cannot be quantified. However, the area under the curve for each fraction showed an increase in both plasma cholesterol and TG (Figure 1 E,F). Therefore, we verified that our rapamycin treatment protocol successful induces hyperlipidemia, comparable to increase in human patients.

### *3.3.2. Rapamycin treatment increases plasma lipids in adipose-specific mTORC1 KO.*

We have previously shown that deletion of mTORC1 in adipose tissue induces hyperlipidemia comparable to rapamycin treatment alone, we wanted to investigate whether mTORC1 adipose KO mice respond to rapamycin treatment. Previous treatments of Raptor<sup>AKO</sup> mice with rapamycin in the random fed state have resulted in various changes in lipids. Since we have shown the hyperlipidemia phenotype in Raptor<sup>AKO</sup> mice is dependent on feeding status, we treated 5-month-old females with rapamycin and measured plasma lipids after fasting and refeeding. For refed plasma cholesterol, TG and NEFA, rapamycin increased plasma lipids comparable to mTORC1 KO alone. However, the combination of rapamycin treatment and loss of adipose mTORC1 further increased plasma lipids compared to either rapamycin or mTORC1 KO alone (Figure 3.2 A-C). In the fasted state however, only plasma TG were increased in rapamycin treated mice not in Raptor<sup>AKO</sup> as previously shown. In addition, we performed FPLC on random fed Raptor<sup>AKO</sup> mice treated with rapamycin on age matched mice from figure 3.1. The plasma cholesterol and TG from the different fractions recapitulates the

total plasma lipids from figure 3.2 A and B (Supplementary Figure 3.1 A). The area under the curve for each fraction is compared to the results from figure 3.1 E and F (Supplementary Figure 3.1 A,C). This suggests that in the fasted state, the effects of rapamycin on plasma lipids is independent of adipose mTORC1. These results indicate that adipocyte mTORC1 inhibition is only partially responsible for the hyperlipidemic effect of rapamycin, and that other factors also play a role.

### 3.3.3. Adipocyte-specific ablation of mTORC2 elevates plasma lipids upon refeeding.

One of the factors that may contribute to rapamycin's effects is the inhibition of mTORC2. In fact, mTORC2 in adipose tissues has also been shown to regulate various aspects of growth and metabolism in response to nutrient and energetic stimuli. Therefore, we tested whether mTORC2 in adipose tissue contributes to rapamycin's effects. To test this, we generate adipose specific rictor knockout mice, we crossed mice in which the *rictor* gene were flanked by loxP sites ( $Rictor^{fl/fl}$ ) to mice expressing cre recombinase under the control of the adipose specific *adiponectin* gene promoter ( $Rictor^{aKO}$ ). As expected, these mice lacked rictor specifically in adipose depots including inguinal, visceral, and brown adipose while maintaining expression in non-targeted tissues such as the liver (Figure 3.3 A). Body weight of  $Rictor^{aKO}$  mice was monitored from 3 to 12 weeks of age. The weight of both male and female  $Rictor^{aKO}$  mice (females no shown) was equal to their littermate controls (Figure 3.3 B). Since  $Raptor^{aKO}$  mice have decreased tissue weights after 12 weeks, we measured tissue weights from 5-month-old males in the refed state. Overall, there was an increase in the weight of the liver in male mice, while the adipose depots were equal to littermate controls (Figure 3.3 C). Interestingly, there was a trend towards decreased fat weight in the inguinal depot

but did not reached significance. To further investigate whether loss of mTORC2 in adipose also induces hyperlipidemia, we measured plasma lipids after an overnight fast and 4 hour refed. Comparable to the mTORC1 adipose KO mice, plasma cholesterol, TG and NEFA were significantly increased in the refed state (Figure 3.3 D-F). In addition, loss of mTORC2 in adipose had no effect on fasting plasma TG levels. Contrary to Raptor<sup>akO</sup> mice, fasting plasma NEFA levels were comparable to littermate controls. We next tested whether the addition of rapamycin treatment would increase plasma lipids in Rictor<sup>akO</sup>. As with Raptor<sup>akO</sup>, rapamycin increased plasma cholesterol and TG to levels comparable to Rictor<sup>akO</sup> alone. However, rapamycin further increased plasma lipids levels in Rictor<sup>akO</sup> compared to rapamycin or Rictor<sup>akO</sup> alone (Figure 3.3 G). These results indicate that loss of mTORC2 signaling in adipose tissue is only partially responsible for the lipid effects of rapamycin treatment.

#### *3.3.4. Rapamycin treatment further increases plasma lipids in adipose-specific mTORC1 and mTORC2 KO mice.*

Since loss of either mTORC1 or mTORC2 in adipose tissue only partially contributes to rapamycin associated hyperlipidemia, we next asked whether inhibition of both complexes is necessary to induce the hyperlipidemia. To investigate whether inhibition of both mTORC1 and mTORC2 accounts for the effects of rapamycin treatment on plasma lipids, we crossed the Raptor<sup>akO</sup> mice with Rictor<sup>fl/fl</sup> mice. Since Raptor<sup>akO</sup> mice develop lipodystrophy after 12 weeks, we used Raptor-Rictor<sup>akO</sup> mice and littermate controls prior to 12 weeks of age. Loss of both complexes in adipose did not affect body weight or blood glucose (Figure 3.4 A,B). Comparable to either KO alone, Raptor-Rictor<sup>akO</sup> had increase plasma cholesterol both in the fasted and refed state, and

increased plasma TG in the refed state (Figure 3.4 C,D). Fasting plasma NEFA were decreased compared to controls, similar to Raptor<sup>aKO</sup> alone. However refed plasma NEFA were increased but did not reach statistical significance. Surprisingly, fasting plasma TG were decreased in Raptor-Rictor<sup>aKO</sup>. Despite that these results indicate that inhibition of both complexes contribute the unrestrained lipolysis caused by rapamycin treatment, loss adipose tissue mTOR signaling only partially contributes to the increased TG effects of rapamycin treatment.

### *3.3.5. Genetic inhibition of lipolysis reverses lipid phenotypes of adipose-specific mTORC1 KO treated with rapamycin.*

We have previously determined that genetic inhibition of lipolysis reverses plasma lipid increases in Raptor<sup>aKO</sup> mice. Previous lipidomics results showed that loss of adipose mTORC1 increases EWAT MG, DG, and TG species. Genetic inhibition of lipolysis in Raptor<sup>aKO</sup> mice (ATGL-Raptor<sup>aKO</sup>), was able to prevent the increase in EWAT DG and TG species, suggesting that the increased lipolysis in Raptor<sup>aKO</sup> mice resulted in increased intracellular DG and TG levels (Figure 3.5 A). However, ATGL-Raptor<sup>aKO</sup> did not prevent the increase in intracellular MG species, suggesting that loss of adipose mTORC1 may alter intracellular lipid processes other than lipolysis (i.e. esterification). We have previously shown that short term inhibition of lipolysis prevents increases in plasma TG in rapamycin treated mice. To investigate whether the confounding effects of long term deleting of adipose mTORC1 accounts for the further increase on plasma lipids with rapamycin treatment, we treated ATGL-Raptor<sup>aKO</sup> mice with rapamycin for 2 weeks. Plasma cholesterol, TG and NEFA levels in Raptor<sup>aKO</sup> mice treated with rapamycin were significantly increased compared to vehicle, rapamycin, ATGL<sup>aKO</sup> +



rapamycin and ATGL-Raptor<sup>aKO</sup> + rapamycin (Figure 3.5 B-D). Interestingly, ATGL<sup>aKO</sup> mice treatment with rapamycin did not significantly increase plasma lipids compared to the vehicle treated mice. Although the ATGL-Raptor<sup>aKO</sup> mice treatment with rapamycin had increased levels compared to vehicle treated mice, plasma lipids were comparable to rapamycin treatment alone. These results suggest that although long term loss of adipose mTORC1 and the concurrent unrestrained lipolysis is responsible for the additive effect of rapamycin on plasma lipids. Whether acute or long term inhibition, how loss of mTORC1 signaling leads to unrestrained lipolysis is still under debate.

### *3.3.6. General mechanism for the widespread effects of Raptor deletion on lipid metabolism.*

We have previously proposed a potential mechanism were suppression of C/EBP $\alpha$  transcriptional activity and decreased perilipin may account for the for the widespread effects of Raptor deletion on lipid metabolism. To test this hypothetical mechanism, we first measured C/EBP $\alpha$  expression. As expected, C/EBP $\alpha$  gene expression is decreased in refed male Raptor<sup>aKO</sup> mice (Figure 3.6 A). We next examined protein expression in IWAT of these mice. In line with gene expression, both p30 and p42 isoforms of C/EBP $\alpha$  protein was decreased compared to littermate controls (Figure 3.6 B,C). This supports our hypothesis that Raptor<sup>aKO</sup> have decreased C/EBP $\alpha$  transcriptional activity and thus decreased expression of lipid metabolism genes regulated by C/EBP $\alpha$ . We have previously shown decreased perilipin gene expression. To investigate whether decreased gene expression results in decreased perilipin protein levels, we measured perilipin proteins expression by western blot from IWAT of Raptor<sup>aKO</sup> mice (Figure 3.6D). Perilipin protein expression was quantified from two different exposures, both

quantification yielded the same decrease in protein levels (Figure 3.6 E). In addition, when lipolysis is stimulated in the fasting condition or with the  $\beta$ 3-adrenergic agonist of CL 316,243, perilipin is phosphorylated. CL 316,243 treatment successfully stimulated phosphorylation of perilipin on Ser522 (Figure 3.6F). However, when Raptor<sup>aKO</sup> mice are treated with CL 316,243, phosphorylation does not increase. This data indicates that the increased lipolysis associated with suppression of C/EBP $\alpha$  transcriptional activity or decreased perilipin expression may contribute to the lipid phenotype of the Raptor<sup>aKO</sup> mice.

### 3.4 Discussion

Rapamycin is known to induce hyperlipidemia in organ transplant patients within 2 weeks of treatment. The doses of rapamycin used to test longevity varies from study to study, ranging from 1.5mg/kg to 4mg/kg when injected and around 2.24mg/kg when encapsulated in food at 14ppm, with some studies using up to 42ppm (Anisimov et al., 2011; Harrison et al., 2009; Miller et al., 2011; Miller et al., 2014; Neff, F. et al., 2013; Zhang, Y. et al., 2014). A lower dose of rapamycin was used in this study, which was able to induce hyperlipidemia within 2 weeks. This hyperlipidemia includes increase in plasma cholesterol, TG, NEFA as well as phospholipids. The increases in total plasma cholesterol and TG is associated with increases in the lipid content of the different lipoproteins, CM/VLDL, the TG-rich lipoproteins, as well as LDL and HDL fractions, in both rapamycin treated animals and Raptor<sup>aKO</sup> mice. Although this does not imply whether production or degradation of these lipoproteins is effected, we have previously demonstrated that production of VLDL by the liver is not increased but rather clearance of TG-rich lipoproteins is decreased.

We first hypothesized that loss of mTORC1 signaling in adipose tissue mediates the effects of rapamycin treatment. However, our mTORC1 adipose KO mice had further increased plasma lipids when treated with rapamycin. Since plasma lipids of rapamycin treated mice are comparable to Raptor<sup>aKO</sup> alone, we speculated that the long term genetic deletion of raptor in these mice is responsible for the exacerbated effects with rapamycin. In fact, when we restored plasma lipids levels of Raptor<sup>aKO</sup> with genetic inhibition of lipolysis (ATGL-Raptor<sup>aKO</sup>), and treated these mice with rapamycin, the additional increase in lipids seen in Raptor<sup>aKO</sup> is prevented, yet lipid levels are still comparable to rapamycin treatment alone. We speculate that pre-existing effects of the loss of adipose mTORC1 prior to rapamycin treatment is responsible for the further increase in plasma lipids seen. It is important to note that ATGL<sup>aKO</sup> mice do not respond to rapamycin. This supports our previous finding that short term lipolysis inhibition with GPR81 agonist (3CI-5OH-BA) prevents the increase in plasma TG seen with rapamycin treatment.

Chronic rapamycin treatment can also impair mTORC2 activity, as mice lacking mTORC2 in adipose tissue have a defect in insulin-dependent suppression of lipolysis. We generated and measured plasma lipids in Rictor<sup>aKO</sup> mice under fast refed conditions to determine if the loss of rictor in adipose tissue had an effect on these parameters. Plasma cholesterol, TG, and NEFA were significantly increased in Rictor<sup>aKO</sup> mice compared to littermate controls in the refed state, while fasted NEFA levels remained the same. This is the only lipid phenotype that differs from our Raptor<sup>aKO</sup> mice. However, mTORC2 adipose KO mice also respond to rapamycin. Kumar et al suggested that both mTOR complexes may act together to fully restrain adipocyte lipolysis (Kumar et al.,

2010). Our results from inhibition of both mTORC1 and mTORC2 in adipose supports this, as all re-fed plasma lipids were significantly increased in this double KO compared not only to control mice but to each KO individually. We speculate that inhibition of lipolysis, using our genetic model or a short term lipolysis inhibitor, would also reverse the increase in plasma lipids in Raptor-Rictor<sup>ΔKO</sup> mice. However, we were unable to test this due to poor breeding associated with rictor genetic models. We did demonstrate that rapamycin treatment in combination with loss of both complexes in adipose tissue still increases plasma lipids. However, this exacerbated effect may be caused from long term loss of mTOR signaling.

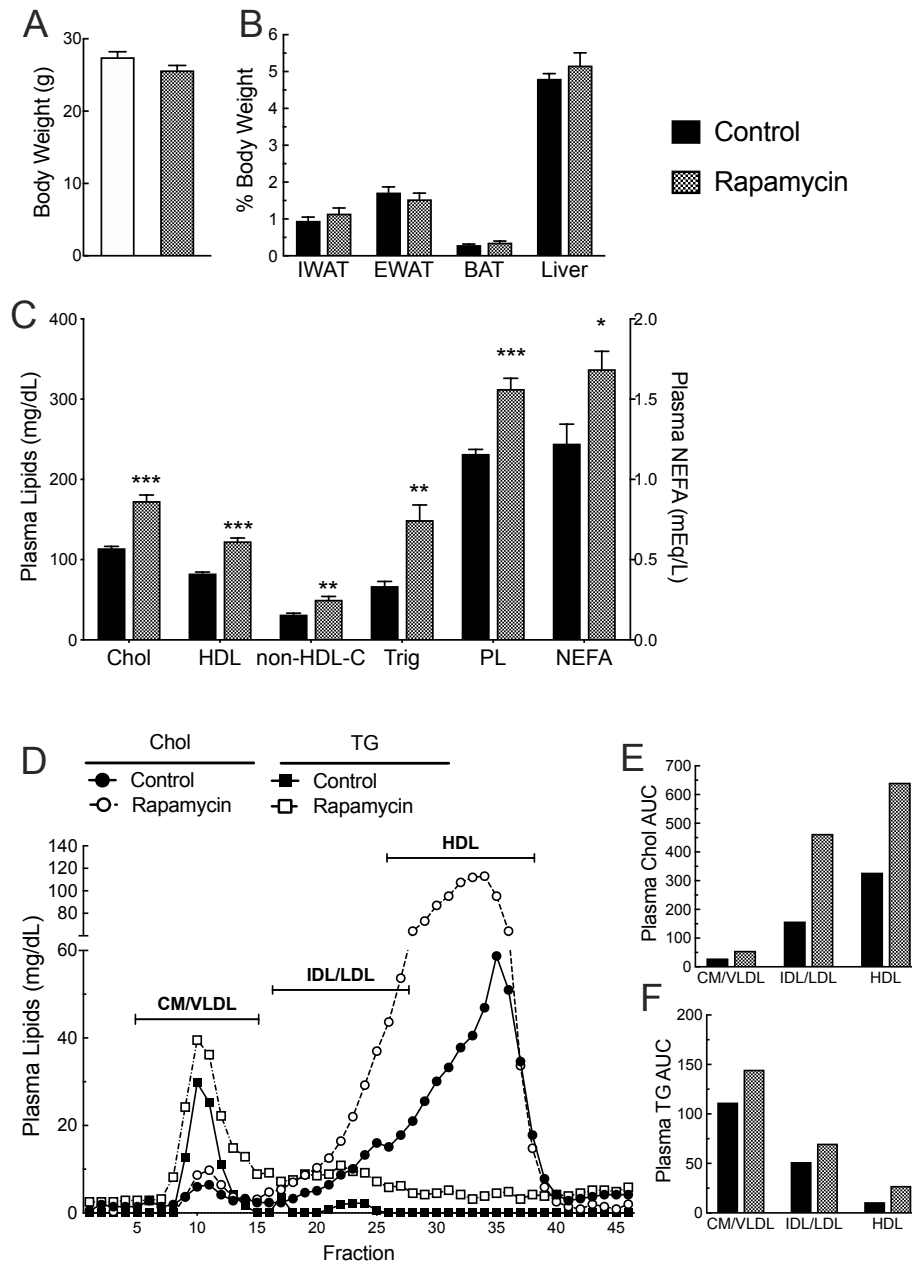
Importantly, it should be noted that rapamycin treatment increased fasting plasma TG levels, where the effect of mTORC1 deletion does not. This suggests an effect of rapamycin on an organ other than adipose in the fasting setting. More investigation, most likely on the muscle is needed to identify the cause of increased fasting plasma TG. In addition, it was recently shown that a rapamycin derivative that avoids mTORC2 inhibition (DL001) also fails to induce hyperlipidemia in fasted mice (Schreiber, K. H. et al., 2019). Therefore, we hypothesize that, in the fed state, the effects of loss of adipose mTORC1 account for the widespread effects of rapamycin on lipid metabolism. This general hypothesis stems from changes in two key lipid metabolism proteins, C/EBP $\alpha$  and its transcription control of key metabolism genes, and perilipin, the lipid droplet protein that coats the droplet layer prevent access to ATGL, the rate limiting enzyme in lipolysis. Here, we show that protein expression for both C/EBP $\alpha$  and perilipin are decreased in mice with loss of adipose mTORC1. Adipose-specific C/EBP $\alpha$  KO mice not only exhibit impaired TG clearance, but develop lipodystrophy (Chatterjee et al.,

2011), although this phenotype develops earlier in life than the lipodystrophy in Raptor<sup>akO</sup> mice. One target of C/EBP $\alpha$  transcriptional control is *Plin1* (encoding perilipin 1). We previously showed decreased gene expression, and have now shown decreased protein expression of perilipin. Decreased perilipin protein leads to an increase in access of ATGL to the lipid droplet, which is normally inhibited in the fed state. As well as decreased levels of protein to be phosphorylated when lipolysis is activated in the fasted state. The latter explains the decrease in fasting plasma NEFA levels in the Raptor<sup>akO</sup> mice. Since adipose perilipin KO mice mimic the lipid phenotype of Raptor<sup>akO</sup> mice, increased basal lipolysis and decreased stimulated lipolysis (Tansey et al., 2001), and mice treated with rapamycin (Pereira et al., 2013) have decreased expression of *Plin*, we propose that in the fed state, rapamycin inhibition of adipose mTORC1 leads to decreased C/EBP $\alpha$  transcriptional activity. This decreased transcriptional activity results in decreased expression of perilipin and subsequent unrestrained lipolysis, leading to hyperlipidemia.

Together, these studies reveal a number of important roles for mTORC1 and mTORC2 in adipose tissue based on feeding status and effects of rapamycin independent of these two complexes in adipose tissue. of suggest that its activation might have a therapeutic effect in metabolic disorders.

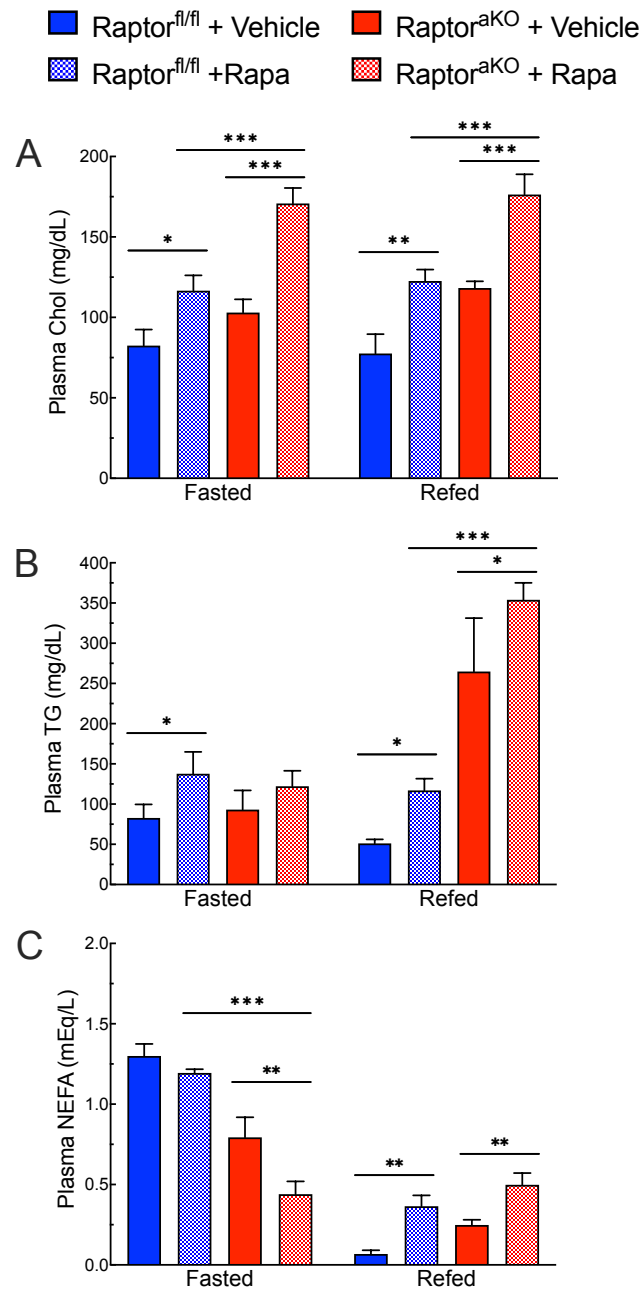
### Figure 3-1. Mice treated with rapamycin for 2 weeks recapitulates the increases in human plasma lipids.

4-month-old male C57BL/6 mice were treated with 2mg/kg rapamycin for 2 weeks to induce hyperlipidemia. (A) Body weight and (B) tissue weights after 2 weeks of vehicle (n=10) or rapamycin (n=11). treatment. (C) Random fed plasma lipids panel from n=6 control and n=8 rapamycin mice. (D). FPLC TG and cholesterol profiles of pooled plasma from the remaining control (n=4) and rapamycin (n=3). (E) Plasma cholesterol and (F) plasma TG AUC. (A-C) Data presented as mean  $\pm$  s.e.m. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. (D-F) Data presented as a single value from pooled samples.



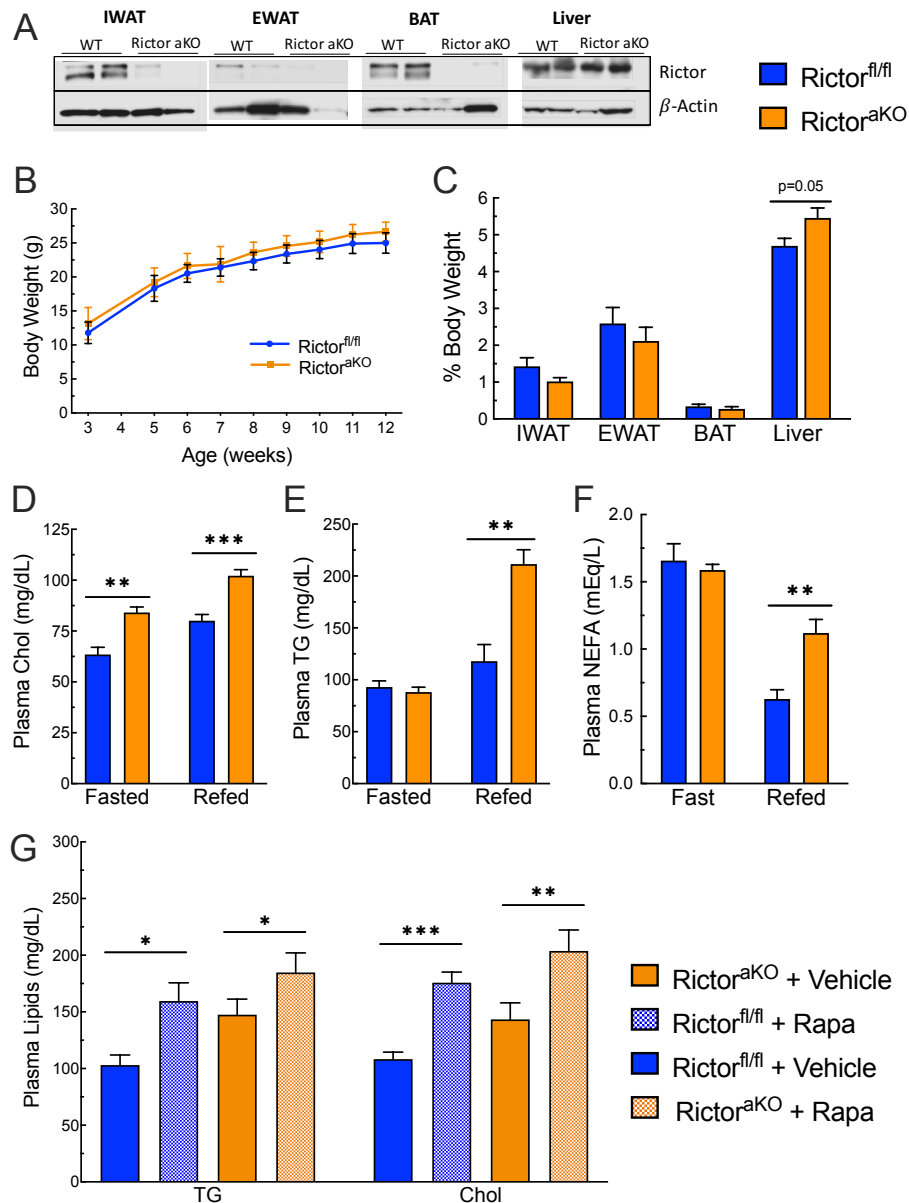
**Figure 3-2. Rapamycin treatment further increases plasma lipids in adipose-specific mTORC1 KO.**

5-month old female  $Raptor^{fl/fl}$  and  $Raptor^{aKO}$  were treated with vehicle or 2mg/kg rapamycin for 2 weeks. (A) plasma cholesterol, (B) plasma TG and (C) plasma NEFA from mice fasted overnight and refed for 4 hours (n=4). Compared to rapamycin treated groups, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 3-3. Adipocyte-specific ablation of mTORC2 elevates plasma lipids upon refeeding.**

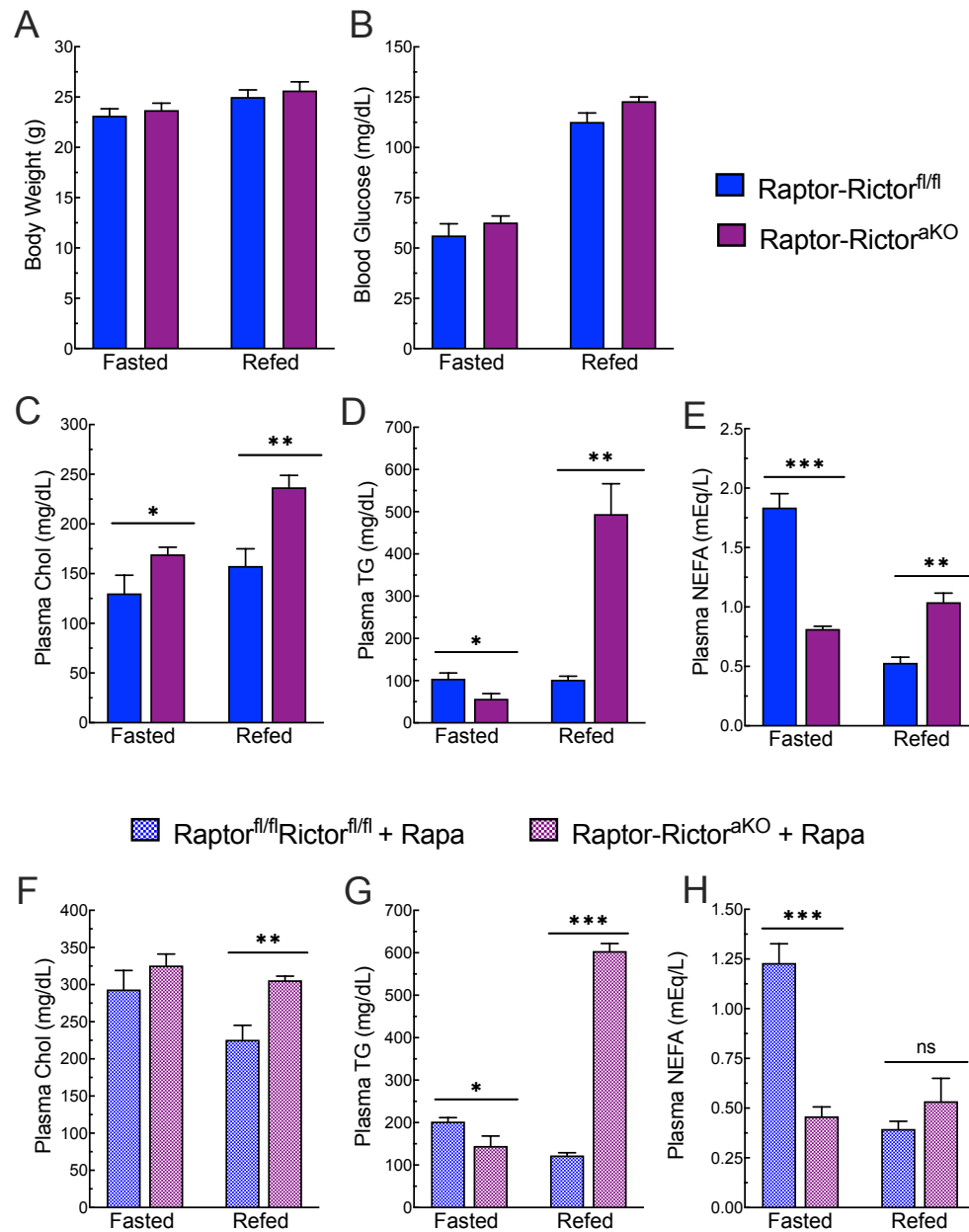
(A) Western blot for Rictor in IWAT, EWAT, BAT and liver. (B) Body weight with age of male Rictor<sup>aKO</sup> compared to Rictor<sup>fl/fl</sup> (n=14). (C) Tissue weights from 5-month-old males. (D) Plasma cholesterol, (E) plasma TG and (F) Plasma NEFA after an overnight fast and refed (n=5). 10-week-old Rictor<sup>fl/fl</sup> and Rictor<sup>aKO</sup> male mice were treated with vehicle or 2mg/kg rapamycin for 2 weeks. (G) Plasma cholesterol and plasma TG from random fed mice after 2 weeks of rapamycin treatment (n=8-10). Data presented as mean  $\pm$  s.e.m. (B-F) \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. (G) Compared to rapamycin treated groups, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.





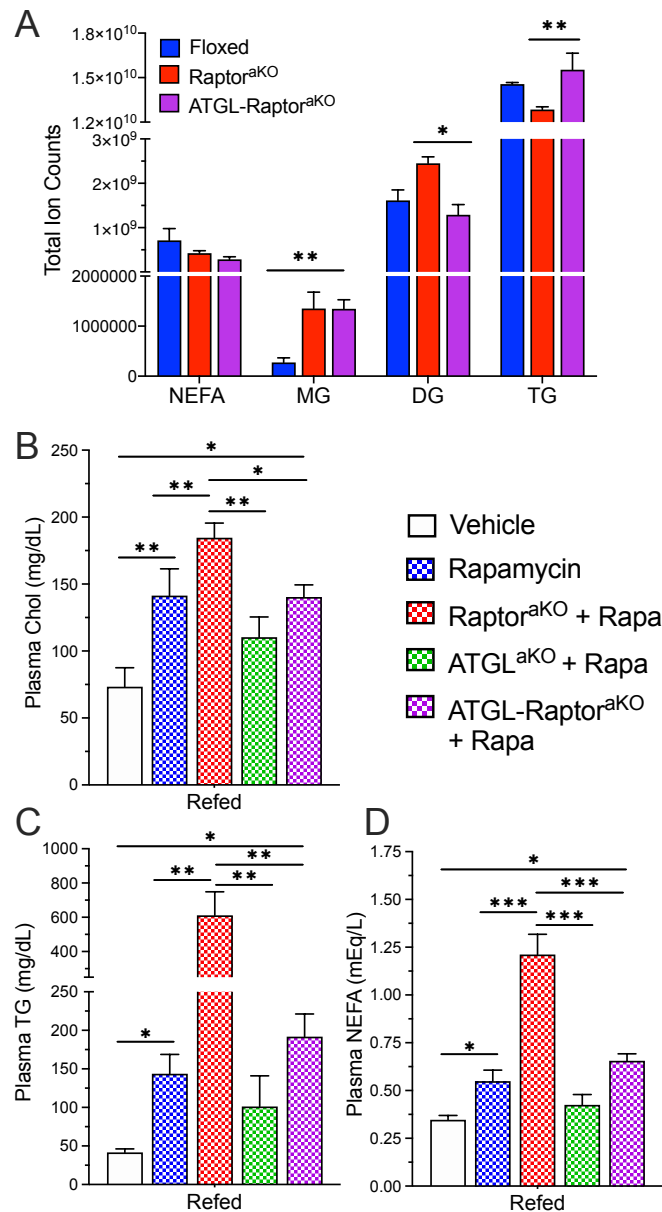
**Figure 3-4. Rapamycin treatment further increases plasma lipids in adipose-specific mTORC1 and mTORC2 KO mice.**

10-week-old Raptor-Rictor<sup>AKO</sup> mice were fasted overnight and re-fed and treated with 2mg/kg rapamycin for 2 weeks. (A) Body weight, (B) blood glucose, (C) plasma cholesterol, (D) plasma TG and (E) plasma NEFA of Raptor-Rictor<sup>AKO</sup> mice compared to floxed littermates (n=4) prior to start of rapamycin treatment. (F-H) Plasma cholesterol, TG and NEFA after 2 weeks of rapamycin. Data presented as mean  $\pm$  s.e.m. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



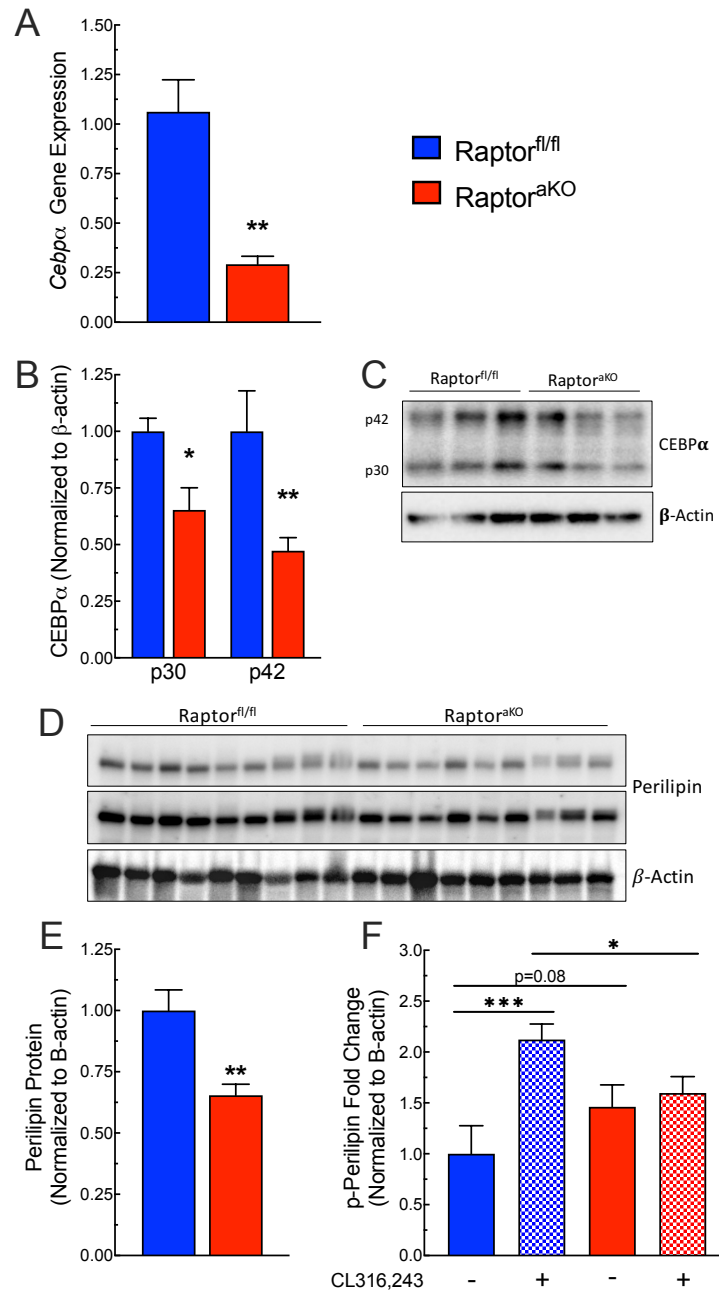
**Figure 3-5. Genetic inhibition of lipolysis reverses lipid phenotypes of adipose-specific mTORC1 KO treated with rapamycin.**

10-week-old male mice were fasted overnight and refed for 4 h prior to collection of EWAT for lipidomics. (E) Total ion counts for NEFA, monoacylglycerols (MG), diacylglycerols (DG), triglycerides (TG) (n=3-5). 8-week-old female mice were treated with vehicle or 2mg/kg rapamycin for 2 weeks. (B) Refed plasma cholesterol, (C) plasma TG and (D) plasma NEFA (n=3-5). Data presented as mean  $\pm$  s.e.m. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



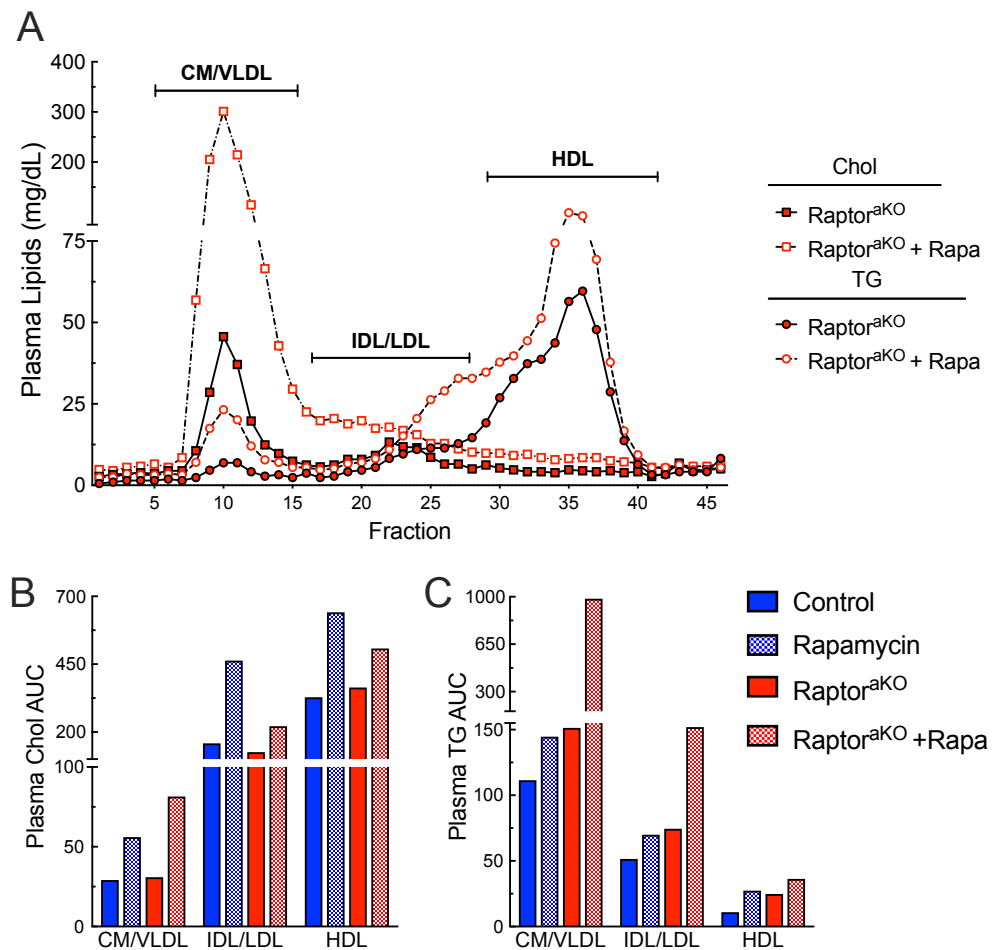
**Figure 3-6. General mechanism for the widespread effects of Raptor deletion on lipid metabolism.**

10-week-old Raptor<sup>akO</sup> mice were treated with vehicle or 2mg/kg rapamycin for 2 weeks. (A) FPLC TG and cholesterol lipoprotein profiles of pooled plasma from random red mice (Raptor<sup>akO</sup> n=10, Raptor<sup>akO</sup> + rapa n=6). (E) Plasma cholesterol AUC and (F) plasma TG AUC. For comparison, the control and rapamycin treated C57BL/6 mice plasma cholesterol and TG AUC is shown. Data presented as a single value



**Supplementary Figure 3-1. Rapamycin treatment further increases lipoprotein lipids in adipose-specific mTORC1 KO.**

4-month-old Raptor<sup>akO</sup> mice were treated with vehicle or 2mg/kg rapamycin for 2 weeks. (A) FPLC TG and cholesterol lipoprotein profiles of pooled plasma from random red mice (Raptor<sup>akO</sup> n=10, Raptor<sup>akO</sup> + rapa n=6). (E) Plasma cholesterol AUC and (F) plasma TG AUC. For comparison, the control and rapamycin treated C57BL/6 mice plasma cholesterol and TG AUC is shown. Data presented as a single value.



## **CHAPTER 4: General Discussion and Conclusions**

## 4.1 Summary of Key Findings

mTORC1 and mTORC2 in adipose tissue, in addition to other metabolic tissues, are known to influence lipid metabolism through multiple pathways. However, the specific cause of hyperlipidemia induced by rapamycin treatment *in vivo* remains undetermined. Here we show that loss of mTORC1 and mTORC2 in adipocytes is sufficient to cause hypertriglyceridemia. Both genetic mouse models displayed increased refeed plasma cholesterol, triglycerides and NEFA levels. The increase in plasma NEFA and TG phenotypes of both Raptor<sup>akO</sup> and Rictor<sup>akO</sup> mice is dependent on the feeding state, providing an explanation for conflicting reports on the effect of rapamycin on NEFA. Other than hyperlipidemia, additional adipose lipid metabolism effects in our Rictor<sup>akO</sup> were not investigated.

Much of this work focused on the role of mTORC1 signaling in adipose. Raptor<sup>akO</sup> mice have decreased LPL gene expression, a small decrease in the LPL activity *in vivo* and intracellularly from adipose tissue. In addition, these mice had delayed clearance of circulating triglycerides. We were able to determine that hepatic production of TG-rich lipoproteins is not a factor contributing to hypertriglyceridemia in our Raptor<sup>akO</sup> mice.

In our Raptor<sup>akO</sup>, we did not observe a significant increase in ATGL protein expression in adipose, yet genetic deletion of ATGL in adipocytes is sufficient to normalize refeed NEFA and TG levels and restored TG clearance without correcting *Lpl* gene expression. We showed that stimulated lipolysis in adipose tissue using a  $\beta$ -adrenergic agonist delayed TG clearance during an oral fat tolerance test. Mechanistically, this work shows defects in adipose mTORC1 signaling induced hypertriglyceridemia is driven mainly by unrestrained lipolysis in the fed state, which likely interferes with lipoprotein lipase activity.

In this study, we hypothesized that inhibition of adipose mTORC1 changes expression levels of two key lipid metabolism proteins, C/EBP $\alpha$  and perilipin, leading to hypertriglyceridemia. We showed that both gene and protein expression were decreased in Raptor<sup>aKO</sup> mice, and these mice fail to phosphorylate perilipin upon stimulation of lipolysis.

The effects of rapamycin on lifespan extension are dose dependent (Bitto et al., 2014; Miller et al., 2014). Therefore, we used a lower dose that has been shown to extend lifespan and mice treated with this dose of rapamycin developed hyperlipidemia, which includes increases in plasma cholesterol, TG, NEFA, phospholipids and in the lipid content of the different lipoproteins. Most importantly, while deletion of either or both mTOR complexes in adipose tissue does not elevate plasma TG levels in the fasted state, rapamycin treatment does.

We successfully show that increased lipolysis with deletion of mTORC1 in adipose tissue induces hypertriglyceridemia. This study also reports a similar effect with rapamycin. Using a GPR81 agonist to suppresses adipocyte lipolysis in rapamycin treated mice, plasma TG levels were normalized after refeeding.

We hypothesized that loss of mTOR signaling in adipose tissue mediates the effects of rapamycin treatment. Plasma lipids were comparable between rapamycin treated mice and our Raptor<sup>aKO</sup> mice and Rictor<sup>aKO</sup> mice. However, when treated with rapamycin, our Raptor<sup>aKO</sup> mice, Rictor<sup>aKO</sup> mice and Raptor-Rictor<sup>aKO</sup> mice had an additional increase in plasma lipids. The additional increase in lipids seen in Raptor<sup>aKO</sup> mice treated with rapamycin was prevented with genetic inhibition of lipolysis (ATGL-Raptor<sup>aKO</sup>). However,

this did not restore levels comparable to wildtype or ATGL<sup>akO</sup> mice treated with rapamycin, which rapamycin did not increase lipid levels in these mice.

#### **4.2 Implications of impaired mTOR in adipose tissue**

It is evident that the adipose tissue is a far more complex tissue than originally described as simply a storage site for excess energy. The integrative responses and lipid regulation within adipocytes can impact metabolic homeostasis. Biological pathways related to adipogenesis, insulin signaling, lipolysis, esterification, and lipogenesis within adipose tissue are implicated as important pathways influencing whole body homeostasis. Both mTOR complexes have been implicated in regulating these pathways, but mainly through unknown mechanisms. Alterations in any part of these pathways ultimately alter the lipid storage of adipose tissue. This leads to excessive circulating levels of fatty acids and ectopic accumulation in non-adipose tissues, such as liver and muscle, which is known to cause metabolic abnormalities. Excessive mobilization of fatty acids into the systemic circulation is responsible for inducing hyperlipidemia, lipodystrophy and insulin resistance.

Recent studies have reported that rapamycin can inhibit the transactivation of several transcription factors such as peroxisome proliferator-associated receptor-gamma (PPAR $\gamma$ ) and CCAAT-enhancer-binding proteins (C/EBP), which play key roles in lipid metabolism and it can change the expression of key enzymes required for fatty acid uptake and triglyceride synthesis in adipose tissue (Houde et al., 2010; Soliman et al., 2010). Adipose-specific C/EBP $\alpha$  KO mice develop lipodystrophy earlier in life than our Raptor<sup>akO</sup> mice, however a decrease in CEBP $\alpha$  transcriptional activity could explain both the lipid phenotype and the development of lipodystrophy. (Chatterjee et al., 2011). As



previously mentioned, Perilipin is a target of C/EBP $\alpha$  transcriptional control. Perilipin adipose KO mice have a comparable phenotype to our Raptor<sup>aKO</sup> of a decrease in adiposity, consistent with perilipin functioning to protect the lipid droplet from basal lipase activity of ATGL (Tansey et al., 2001). Additionally, without adipose Perilipin, CGI-58 is readily available to co-activate ATGL, further increasing lipolysis. In addition, adipocytes deficient in perilipin have a blunted response to isoproterenol stimulation of lipolysis (Kim, S. J. et al., 2016). Therefore, this results from this work suggest that the decreased levels of the perilipin protein itself on the lipid droplet may account for the unrestrained lipolysis in the refed state and the lack of protein results in decreased phosphorylation and thus stimulation of lipolysis as seen by decreased plasma NEFA in the fasting state.

In the fed state, intracellular adipose lipase activity is low, and the direction of NEFA transport is inward, promoting efficient uptake of LPL-generated fatty acids. Presumably, the vast majority of these fatty acids taken up by adipose tissue are directed toward esterification and ultimately triglyceride storage. Efficient storage of dietary fat requires adequate suppression of adipose tissue lipolysis, which typically depends on the antilipolytic effects of the increased insulin concentrations that are observed after refeeding. Abnormally high NEFA releases in adipose tissue could contribute to increased VLDL triglyceride production by raising postprandial portal vein NEFA concentration (Lewis, 1999; Miles et al., 2005). In addition to insulin's suppression of adipose lipolysis, insulin activates LPL transcriptionally and its secretion to the capillary endothelium (Wang, H. & Eckel, 2009). While it is well established that insulin regulates mTORC1 activity, mTORC1 seems to regulate LPL activity through other mechanisms intendent of insulin. We do see a decrease in LPL mRNA in white adipose tissue,

consistent with the insulin dependent regulation on LPL transcription. A significant decrease in post-heparin LPL activity was observed in patients showing sirolimus-induced hypertriglyceridemia and that gene expression was decreased by 60% in the presence of rapamycin (Hoogeveen et al., 2001; Kraemer et al., 1998). In addition, treatment with rapamycin associated with a 92% increase in apolipoprotein CIII, which is a potent inhibitor of LPL (Morrisett et al., 2002). Thus, all these data indicate that mTOR inhibitors are likely to decrease LPL activity and thus to reduce the catabolism of triglyceride-rich lipoproteins leading to hypertriglyceridemia. However, the effect on secretion and hydrolysis activity with loss of mTORC1 signaling is not altered in our study and the clinical assessment of rapamycin was performed in the fasted state, where LPL activity is typically suppressed in adipose. Instead, the indirect effects of unrestrained lipolysis in the fed state clearly inhibit the physiological role of LPL to remove triglycerides from lipoproteins. It has long been appreciated that NEFA can suppress LPL through multiple mechanisms, including product inhibition, displacement of the active enzyme from the endothelium, and preventing its interaction with activating factors such as ApoC-II (Amri et al., 1996; Gordts et al., 2016; Peterson, J. et al., 1990; Saxena et al., 1989). As demonstrated by the use of stimulating NEFA releases from adipose tissues during a triglyceride clearance assay, the increase in circulating NEFA decreases the rate of clearance of TG from the blood for proper storage in adipose tissue. This key finding, which has been shown by a few previous groups, has not been extensively studied in the clinical setting. In some cases of hypertriglyceridemia, inhibiting adipose lipolysis may aid in treatment for patients. In the context of anti-ageing agents or treatments that target mTORC1, development of inhibitors need to be assessed for the effect on adipose lipolysis.

One factor that can contribute to impaired lipid clearance is lipodystrophy, in which there is less total adipose tissue. This may contribute to the worsening of mTORC1 phenotypes with age, but we do not believe it drives the basal phenotype because we see the development of hypertriglyceridemia prior to the onset of lipodystrophy. We do believe that the increased lipolysis leads to lipodystrophy in our mice. Mice with an inability to maintain adipose-depot stores have profound changes in their systemic physiology, exhibiting similar effects as human patients with lipodystrophy (Garg et al., 2004). The failure to maintain adipose stores due to this enhanced lipolysis progressively decreases adipose mass. However, when downstream factors in the insulin signaling cascade are targets, mild lipodystrophy phenotypes are observed with surprisingly profound effects on insulin sensitivity. Although insulin resistance was not the focus of this study, other groups have shown that ablation of the mTORC1's function also leads insulin intolerance and progressive lipodystrophy. This suggests that mTORC1 might be a critical downstream target of insulin signaling in the maintenance of adipose tissue (Lee, P. L. et al., 2016; Liu, D. et al., 2016). However, this group did not assess insulin tolerance prior to the onset of lipodystrophy. Therefore, it remains unclear whether unrestrained lipolysis leads to lipodystrophy or if insulin intolerance is the cause. In addition, the effect of increased lipolysis most likely leads to the insulin phenotype of Raptor adipose KO mice, suggesting a possible mechanism that increased lipolysis may lead to insulin resistance which causes lipodystrophy.

In mTORC2 adipose KO mice, insulin resistance and a progressive lipodystrophy is also seen (Tang et al., 2016). This mouse model also exhibits defects in glucose uptake, regulation of *de novo* lipogenesis, and an inability to fully suppress lipolysis in response

to insulin (Tang et al., 2016). Suggesting that the role of mTORC2 in rapamycin's insulin side effects may play a role. However, the inhibition of mTORC1 is mainly responsible for the lipodystrophy induced by excess lipolysis, as reverse the lipolysis phenotype in Raptor<sup>akO</sup> mice prevents the loss of adipose mass.

#### **4.3 Implications of rapamycin on adipose mTOR signaling**

The pathophysiology of mTOR inhibitor-induced dyslipidemia is not yet totally clear. In vitro studies have shown that mTOR inhibition by rapamycin increases fatty acids in hepatocytes oxidation while decreasing fatty acid flux into anabolic storage pathways and skeletal muscles (Brown, N. F. et al., 2007; Busaidy et al., 2012; Sipula et al., 2006). In addition, several studies have shown that rapamycin decreases the gene expression of lipogenic enzymes such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase (Brown, N. F. et al., 2007; Mauvoisin et al., 2007; Peng, T. et al., 2002). The work from this study and previous studies indicate that mTOR inhibitors are likely to decrease LPL activity and thus to reduce the catabolism of triglyceride-rich lipoproteins leading to hypertriglyceridemia. However, most studies conclude that the effect on LPL is a direct inhibition of activity by apolipoproteins or by decreased expression through the insulin pathway. One study did find no significant differences in post heparin LPL activity with rapamycin and concluded that rapamycin on lipid metabolism is most likely not mediated by LPL (Hoogeveen et al., 2001). However, these clinical studies on patients treated with rapamycin are all performed in the fasted state. In 2007, Kimak et al. did a fasting and refeeding study in renal transplant patients but not focused on specifically on the use of rapamycin as the immunosuppressant (Kimak et al., 2007). This study found a ~40 mean increase in plasma triglycerides from fasting to refeeding. This is in line with

our results, that the refeed state is important when measuring lipids in the context of rapamycin inhibition of adipose mTOR signaling.

While most of the beneficial effects of rapamycin in regards to health and longevity are due to inhibition of mTORC1, many of the negative side effects are mediated through the off-target inhibition of mTORC2 with rapamycin. Therefore, it is believed that the side effects of rapamycin and rapalogs can be avoided by selectively inhibiting mTORC1 signaling (Lamming, 2014). In 2019, DL001, a rapamycin analog that has greater selectivity for mTORC1 compared to rapamycin, selectively inhibits mTORC1 both in *in vitro* and *in vivo* and does not inhibit mTORC2. Schreiber et al. show that DL001, unlike rapamycin, does not affect glucose or lipid homeostasis. DL001 did not increase blood levels of cholesterol, triglycerides, and free fatty acids. In white adipose tissues, which rapamycin increases ATGL protein expression, DL001-treated animals only had an 16% increase in ATGL. Similarly to our data, this group concluded that mTORC1 regulates lipolysis in adipose tissue, however, they suggest that this effect is not sufficient to cause hyperlipidemia induced by rapamycin. This study, much like many clinical studies, only examines fasted lipids. While rapamycin does increase fasting triglycerides, which is independent of adipose mTORC1 according to our data, triglyceride levels are increased more substantially upon feeding. Adipose mTORC1 signaling is significantly decreased during fasting. Therefore, all studies examining rapamycin or mTOR inhibitors in the fasted state are excluding potential results due to the influence of the adipose tissue on whole body lipid homeostasis, specifically the refeed regulation on intracellular lipolysis and LPL.

The study on DL001 was performed on mice after 5 weeks of treatment, which is longer than most clinical studies. The results indicate that even if this compound increases refeed adipose lipolysis and plasma triglycerides as in our study, the plasma levels of these lipids are normalized upon fasting. However, as previously discussed, long term increase in lipolysis can lead to lipodystrophy and insulin resistance, leading to potential complications with long term use of DL001 or other compounds. It remains unlikely that compounds that inhibit mTOR signaling can be used clinically for a long term without an additional therapeutic agent, such as lipolysis inhibitors. Although niacin has been used clinically to suppress postprandial hypertriglyceridemia in humans, may side effect occur after long term use, such as flushing (Montserrat-de la Paz et al., 2018; Usman et al., 2012). In this study, the use a GPR81 agonist, which inhibits lipolysis specifically in adipose tissue, successfully suppressed NEFA release from adipose tissue and sufficiently normalize plasma TG in rapamycin-treated mice. The use of rapamycin, rapalogs or other mTOR inhibitors that increase lipolysis in conjunction with a GPR81 agonist may be sufficient to improve lifespan while eliminating hyperlipidemia for long term use in humans. However, long term side effects of the GPR81 agonists have yet to be explored.

#### **4.4 Limitations of This Study**

In chapter 3, appropriate parameters, male mice less than 12 weeks old were fasted and refeed, were used for all experiments. However, there are a few limitations of this study that must be addresses from chapter 4.

Although multiple cohorts of Raptor<sup>akO</sup> mice less than 12 weeks old were treated with rapamycin, random fed plasma lipids results were variable from cohort to cohort. We

suspect this discrepancy is due to the later observation of feeding status on plasma lipids in Raptor<sup>akO</sup> mice. For Figure 3.2, mice older than 12 weeks of age were used to measure fasted and refed plasma lipids with rapamycin treatments. Therefore, it should be noted that those mice have developed lipodystrophy prior to the start of rapamycin treatment.

While Rictor<sup>akO</sup> mice do not develop lipodystrophy with age, it should be noted that the mice used in Figure 3.3 A-F are around 5 months old. In Figure 3.3, plasma lipids were measured in the random fed state. Although only results from one cohort is graphed, this experimental design was repeated with multiple cohorts. Due to progressively poor breeding with the Rictor<sup>fl/fl</sup> line that occurred, we were unable to obtain a cohort with enough mice to measure fasted and refed plasma lipids with rapamycin treatments.

Similarly to the breeding issues with the Rictor<sup>fl/fl</sup> line, the Raptor-Rictor<sup>fl/fl</sup> line failed to produce litter sizes with enough mice of each genotype for experiments. In Figure 3.4, only one cohort of Raptor-Rictor<sup>akO</sup> mice were treated with rapamycin and no mice were available for vehicle treated groups. Therefore, the cohort used in Figure 3.4 A-E is pre-rapamycin treatment. Figure 3.4 F-H is 2 weeks post-treatment of the same cohort. Due to the lack of vehicle treatment, the tissues weights and downstream signaling (both gene and protein expression) phenotypes of Raptor-Rictor<sup>akO</sup> mice independent of rapamycin were not investigated.

#### **4.5 Future Directions**

Although this study provides new understanding of the influence of mTOR signaling in adipose tissue on lipid homeostasis and the development of hypertriglyceridemia with rapamycin, there are questions that still remain unanswered. In regards to rapamycin

treatment: 1. What are the effects on plasma TG clearance and hepatic TG secretion? 2. What are the effects on LPL activity in both the fasted state and refed state? 3. How do these effects of rapamycin treatment compare to the changes in Raptor<sup>akO</sup>? These questions can be answered by performing oral fat tolerance tests, hepatic TG secretion assay and by measuring fasting and refed LPL activity (gene expression, intracellular LPL activity and post heparin plasma LPL activity in mice treated with rapamycin. We can determine if production or degradation of plasma TG results in hyperlipidemia. These results would also identify if the results from our Raptor<sup>akO</sup> mice are rapamycin sensitive or insensitive effects. It is hypothesized that rapamycin will delay TG clearance as a result of mTOR inhibition in the adipose and that the cause of increased fasted plasma TG, which is independent of mTORC1 in adipose tissue, is not due to increased hepatic TG secretion but rather a defect in muscle LPL activity. Performing these experiments on Rictor<sup>akO</sup> mice can also improve our understanding of adipose mTORC2 signaling on lipid homeostasis.

The known downstream signaling of mTORC1 and mTORC2 in adipose tissue does not adequately explain the detrimental effects on lipid metabolism. Therefore, a better understanding of the signaling pathways is needed. Using mass-spectrometry-based phosphoproteomics we can extend mTOR signaling pathway, allowing identification of potential targets that mediate lipid metabolism regulation by adipose mTOR. Phosphoproteomics has been used to investigate the roles of mTOR complexes in liver, but in the adipose tissue, this technique has generally lagged. This is in part due to the difficulty of sufficiently removing fat from the tissue. Unwanted cellular material, such as lipids and genomic DNA, results in signal suppression. Removal of lipids



provides a cleaner, clearer spectrum of peptides. Proteins embedded in lipid bilayers require efficient and complete digestion, which remains a challenge and an area for continuing methodological development (Shaik et al., 2016). As adipose tissue is mainly comprised of lipids, a successful and universal method for phosphoproteomics on white adipose tissue has yet to be established. Phosphoproteomics will allow an unbiased and quantitative analysis to determine new targets downstream of mTOR signaling in adipose tissue. We have collaborated with the Garcia lab here at Penn to establish a robust method for performing mass spectrometry on this tissue. Our ongoing collaboration with Ben Garcia's mass spectrometry group has already established a robust method for performing mass spectrometry on white adipose tissue in which that we can detect several thousand distinct phosphosites. By treating Raptor<sup>AKO</sup> mice and Rictor<sup>AKO</sup> with insulin, which regulates lipolysis and LPL in adipose, or CL316,243, which regulates independently from insulin signaling and with different downstream effects, we can identify the targets of lipid metabolism that are regulated by mTOR signaling. In addition, performing phosphoproteomics on white adipose tissue from rapamycin treated mice will identify rapamycin sensitive and insensitive targets that are downstream of either mTOR complex.

Not only does this work expand our understanding of the role of adipose mTOR complexes in the propagation of hyperlipidemia, but also provides insight on the impact adipose tissue lipid metabolism has on the development of lipodystrophy and insulin resistance. The results from this study and the future work has the potential of revealing new biology and potentially new therapeutic strategies to combat hyperlipidemia associated with rapamycin treatment, allowing therapeutic use as an anti-ageing agent in humans, as

well as adipose induce metabolic dysfunction. thus informing our understanding of the role of adipose mTOR complexes in the propagation of insulin resistance from the adipocyte to the muscle and liver.

## **CHAPTER 5: Materials and Methods**

## 5.1 Animals and Treatments

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Adipocyte-specific raptor knockout mice ( $Raptor^{akO}$ ) were generated by crossing raptor floxed mice containing loxP sites flanking exon 6 of the targeted *Rptor* (B6.Cg- $Rptor^{tm1.1Dmsa}/J$ ) with mice containing the Adipoq-Cre BAC transgene express Cre recombinase under control of the mouse adiponectin (Adipoq) promoter/enhancer regions (B6.FVB-Tg(Adipoq-cre)1Evdr/J) (Eguchi et al., 2011; Sengupta et al., 2010). ATGL- $Raptor^{akO}$  were generated by crossing ATGL<sup>fl/fl</sup> mice containing loxP sites flanking exons 2-7 of the *Pnpla2* gene (B6N.129S-Pnpla2<sup>tm1Eek/J</sup>) (Sitnick et al., 2013). with  $Raptor^{akO}$  mice. To generate adipose specific rictor knockout mice, we crossed mice in which the *rictor* gene were flanked by loxP sites ( $Rictor^{fl/fl}$ ) to mice expressing cre recombinase under the control of the adipose specific *adiponectin* gene promoter ( $Rictor^{akO}$ ) (Guertin et al., 2009). The following primer sequences were used for genotyping: *Rptor*: forward, 5'-CTCAGTAGTGGTATGTGCTCAG-3'; reverse, 5'-GGGTACAGTATGTC AGCACAG-3'. *Pnpla2*: forward 5'-GAGTGCAGTGTCTTCACCA-3'; reverse 5'-ATCAGGCAGCCA CTCCAAC-3'. *Rictor*: forward 5'-GACACTGGATTACAGTGGCTTG-3'; reverse 5'-ACTGAATATGTTTCATGGTTGTG-3'. Adipoq-Cre: forward 5'-ACCTCCTGGGAGAGTGAGGGC-3'; reverse 5'-GCATCGACCGGTAATGCAGGC-3'. Age-matched floxed mice ( $Raptor^{fl/fl}$ ,  $Raptor^{fl/fl}$  ATGL<sup>fl/fl</sup>, ATGL<sup>fl/fl</sup>),  $Raptor^{akO}$ , ATGL<sup>akO</sup> and ATGL- $Raptor^{akO}$  male mice were studied between 10 weeks to 12 weeks of age unless otherwise stated. For fasting and refeed experiments, mice were fasted overnight and refed for 4 h. For stimulating lipolysis, mice were injected intraperitoneally with 1 mg/kg  $\beta$ 3-adrenergic receptor agonist, CL316,243 (Sigma-Aldrich). For rapamycin treatment,

mice were intraperitoneally injected with 2 mg/kg rapamycin (LC Laboratories) in 0.25% PEG400/Tween80 daily for 2 weeks. To suppress lipolysis, mice were intraperitoneally injected with 100 mg/kg GPR81 Agonist, 3CI-5OH-BA (Sigma-Aldrich), at the beginning of refeeding. Mice were sacrificed by cervical dislocation after an overnight fast and 4 h of refeeding.

## *5.2 Western Blot*

Tissue samples were lysed in RIPA buffer supplemented with 10  $\mu$ L/mL Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher, 78440), and homogenized using a TissueLyser II (Qiagen). Lysates were incubated with gentle rocking for 30 min at 4°C then centrifuged 12,000 g for 20 minutes at 4°C twice, transferring the supernatant. Protein concentration was determined by BCA Assay (Pierce). 12-20  $\mu$ g of total protein was loaded onto sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 4-15% Tris-Glycine gradient gels or 10% resolving gels (Bio-Rad) and subsequently transferred to PVDF membranes (Bio-Rad). Membranes were blocked with either 3% BSA (for phospho-proteins) or with 3% non-fat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% tween 20) and incubated overnight at 4 °C in blocking solution with the following primary antibodies at 1:2000: Raptor (Cell Signaling, 2280), ATGL (Cell Signaling, 2138), pAkt Ser473 (Cell Signaling, 9271), Akt (Cell Signaling, 9272), pS6 Ser240/Ser244 (Cell Signaling, 2215), S6 (Cell Signaling, 2212), p4EBP1 Ser65 (Cell Signaling, 9451), 4EBP1 (Cell Signaling, 9452), Rictor (Cell Signaling, 2140), Perilipin (NB110-40760), CEBP $\alpha$  (Santa Cruz, sc-61) and pPerilipin Ser522 (Vala Sciences, 4856). Secondary HRP conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc.) were used at 1:10,000 in TBST and 1:10,000 for HRP conjugated B-

actin (Abcam, 9620272). Immunolabeling was detected using the PICO Plus or Femto ECL reagent (Pierce). Band densities were quantified using Image Lab version 5.2.1 and band intensities were normalized to  $\beta$ -actin (Bio-Rad).

### *5.3 Plasma Lipid Analysis*

Blood was collected from the tail veins of mice in Microvette CB300 LH K2E tubes (Sarstedt). For retro orbital bleeding, mice were first anesthetized with isoflurane. Blood was collected into Fisherbrand Micro Blood Collecting Tubes, Natelson; Ammonium heparin. Plasma was analyzed for cholesterol using the Infinity<sup>TM</sup> Cholesterol Liquid Stable Reagent (ThermoFisher, TR13421), triglycerides using the Infinity<sup>TM</sup> Triglycerides Liquid Stable Reagent (ThermoFisher, TR22421), non-esterified fatty acids using the Free Fatty Acid Kit (Wako, HR series NEFA-HR(2)) and glycerol using the Free Glycerol Reagent (Sigma, F6428) according to the manufacturer's instructions. For plasma lipid panels on rapamycin treatment mice, lipids were measured using enzymatic assays as described (Amar et al., 1998; Dugi et al., 2000; Nong et al., 2003). HDL-cholesterol (HDL-C), was determined after precipitation of ApoB-containing lipoproteins with dextran sulfate (Bayer Corp., Tarrytown, NY), and then subtracted from total cholesterol (TC) to obtain non-HDL cholesterol (Gonzalez-Navarro et al., 2004). Lipoproteins were analyzed by fast-protein liquid chromatography (FPLC), using 150  $\mu$ l of pooled plasma as described (Amar et al., 1998; Dugi et al., 2000; Gonzalez-Navarro et al., 2004).

### *5.4 Tissue Triglycerides*

Tissue pieces were homogenized in a 20x volume of 2:1 chloroform:methanol then incubated at room temperature with agitation for 3 hours. After addition of 0.9% NaCl, samples were vortexed and centrifuged (2000 rpm, 10 minutes). The lower organic

phase was collected and dried under N<sub>2</sub> gas. TG content was measured using the Infinity™ Triglycerides Liquid Stable Reagent (ThermoFisher, TR22421) and normalized to starting tissue weight.

#### *5.5 Hepatic TG Secretion*

Mice were either fasted for four hours or overnight and refed for 4 hours and injected intraperitoneally with 1 g/kg body weight Pluronic F-127 (P-407) (BASF, 30085239) in PBS. Blood was drawn at indicated time points after P-407 injection. 2 mL/kg blood was collected at each time point. Plasma triglycerides and NEFA were measured. For CL316,243 treatment, mice were injected 1 hour post P-407 injection.

#### *5.6 Oral Fat Tolerance Test*

Mice were fasted overnight and fed 2 mg/g body weight Olive oil (Acros, 8001-25-0) through oral gavage. Approximately 50 µL of blood was collected at baseline (prior to gavage) and at indicated time points. Plasma triglycerides and NEFA were measured. For CL316,243 treatment, mice were injected 1 hour post gavage.

#### *5.7 Quantitative Real Time RT-PCR analysis*

Total RNA was extracted using TRIzol. RNA was used to generate cDNA with the High Capacity Reverse Transcription Kit (Applied Biosystems). Reactions were run on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems) with results normalized to *Tbp*. Primer sequences are listed in Table 5.1.

### *5.8 Post Heparin Plasma LPL*

Mice were either fasted for four hours or overnight and refed for 4 hours then injected with 0.5 units/g body weight (4  $\mu$ L/g of 125 units/ml in sterile PBS) heparin (Sagent, 25021-400-10) through the tail vein. Mice were bled retro-orbitally at baseline and then 10 minutes after heparin injection. Plasma LPL activity was assayed using the Lipoprotein Lipase (LPL) Activity Assay Kit (Fluorometric) (Cell Biolabs, STA-610) according to the manufacturer's instructions.

### *5.9 Tissue LPL Activity*

Tissues (IWAT, EWAT, BAT, skeletal muscle) were assayed using the Lipoprotein Lipase (LPL) Activity Assay Kit (Fluorometric) (Cell Biolabs, STA-610) according to the manufacturer's instructions.

### *5.10 Lipidomics*

Liquid chromatography mass spectrometry (LC-MS) analysis of lipids from mice adipose samples was performed as follows. About 30 mg of frozen tissue samples were weighed and then pulverized in a CryoMill (Retsch, Germany) with stainless ball at liquid nitrogen temperature. 1 mL 0.1M HCl in 50:50 methanol:H<sub>2</sub>O was added to the tissue powder, vortexed for 10 seconds, and let sit in -20°C freezer for 30 min. Then 0.5 mL of chloroform was added to the mixture and vortex to mix well, and let sit on ice for 10 min. Samples were centrifuged at 13200 rpm for 10 min and the chloroform phase at bottom was transferred to a glass vial as the first extract using a Hamilton syringe. 0.5 mL chloroform was added to the remaining material and the extraction was repeated to get the second extract. The combined extract was dried under nitrogen flow and re-



dissolved in solvent of 1:1:1 methanol:chloroform:2-propanol using a ratio of 1 mL of solvent per 10 mg of initial tissue weight. Fatty acids and lipids were analyzed on a Q Exactive Plus mass spectrometer coupled to vanquish UHPLC system (ThermoFisher Scientific, San Jose, CA). Each sample was analyzed twice using same LC gradient but different ionization mode on mass spectrometer to cover both positive charged and negative charged species. The LC separation was achieved on an Agilent Poroshell 120 EC-C18 column (150 x 2.1 mm, 2.7  $\mu$ m particle size) at a flow rate of 150  $\mu$ L/min. The gradient was 0 min, 25% B; 2 min, 25% B; 4 min, 65% B; 16 min, 100 %B; 20 min, 100% B; 21 min, 25% B; 27 min, 25% B (Papazyan et al., 2016). Solvent A is 1mM Ammonium acetate + 0.2% acetic acid in Water:Methanol (90:10). Solvent B is 1mM Ammonium acetate + 0.2% acetic acid in Methanol:2-propanol (2:98). For all experiments, 5  $\mu$ l of extract was injected with column temperature set to 60 °C. The Q-Exactive Plus mass spectrometer was operated scanning m/z 70-1000 with a resolution (at m/z 200) of 140,000. MS parameters are as follows: sheath gas flow rate, 28 (arbitrary units); aux gas flow rate, 10 (arbitrary units); sweep gas flow rate, 1 (arbitrary units); spray voltage, 3.3 kV; capillary temperature, 320°C; S-lens RF level, 65; AGC target, 3E6 and maximum injection time, 500 ms. Data analyses were performed using MAVEN software which allows for sample alignment, feature extraction and peak picking (Melamud et al., 2010). Exacted ion chromatogram for each metabolite was manually examined to obtain its signal, using a custom-made metabolite library.

#### *5.11 Ex vivo Lipolysis*

Epididymal adipose tissue was isolated from random fed mice. Each fat pad was cut into equal size pieces and were placed in a 96 well plate containing 150  $\mu$ L of phenol-free

DMEM media (Thermo Fisher Scientific, 21063) supplemented with 2% fatty acid free bovine serum albumin (BSA) (Sigma, A8806). For mTORC1 inhibition, 500nM rapamycin was added to media. For ATGL inhibition, 100 $\mu$ M ATGLinhibitor (MedKoo, 510273) was added to media. Tissue pieces were pre-incubated for 60 min. For basal lipolysis, tissue pieces were incubated in new media for 60 minutes. For stimulated lipolysis tissue pieces were pretreated with 1 $\mu$ M CL316,243 for 30 min. To detect release of NEFA, tissue pieces were then placed in a new well with same media for 60 min. Media was measured for NEFA and normalized to protein. To determine protein concentration, each piece was incubated with 1mL chloroform for 60 minutes. Then the tissue was transferred and lysed in 0.3M NaOH containing 0.1% sodium dodecyl sulfate (SDS) and incubated overnight at 65°C. Protein concentration was determined by BCA Assay.

#### *5.12 Statistical analysis*

All data are presented as mean  $\pm$  SEM and analyzed using Prism version 8 (Graphpad). Statistical significance was determined using unpaired two-tailed Student's t-test for single comparisons and one-way or two-way ANOVA followed by Tukey posthoc test for multiple comparisons unless otherwise indicated.

**Table 5-1. Primer Sequences for Quantitative Real Time RT-PCR**

<b>Gene</b>	<b>Forward Primer 5'-3'</b>	<b>Reverse Primer 5'-3'</b>
<i>Angptl4</i>	AAGATGCACAGCATCACAGG	ATGGATGGGAAATTGGAGC
<i>Arg1</i>	CCAGGGACTGACTACCTTAAAC	GAAGGCGTTTGCTTAGTTCTG
<i>CD11c</i>	ATGTTGGAGGAAGCAAATGG	CCTGGGAATCCTATTGCAGA
<i>CD45</i>	ATATCGCGGTGTAAAACTCGTC	TAGGCTTAGGCGTTTCTGGAA
<i>CD68</i>	TGCCACAGTTTCTCCACCA	AAGAGCCCCCTTGGACCTTG
<i>Cgi-58</i>	TGGTGTCCCACATCTACATCA	CAGCGTCCATATTCTGTTTCCA
<i>F4/80</i>	TGGCTGCCTCCCTGACTTTC	CAAGATCCCTGCCCTGCACT
<i>Il-6</i>	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
<i>Lpl</i>	ACTCGCTCTCAGATGCCCTA	TTGTGTTGCTTGCCATTCTC
<i>Plin1</i>	AGGCTGTCTCCTCTACCAAA	CCACAGTGTCTACCACGTTATC
<i>Tnfa</i>	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
<i>Cebp<math>\alpha</math></i>	AAACAACGCAACGTGGAGAC	TGTCCAGTTCACGGCTCAG
<i>Tbp</i>	CCCCTTGTACCCTTCACCAAT	GAAGCTGCGGTACAATTCCAG

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